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Targeting the oncogenic m6A demethylase FTO suppresses tumourigenesis and potentiates immune response in hepatocellular carcinoma

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► Additional supplemental material is published online only. To view, please visit the journal online (<https://doi.org/10.1136/gutjnl-2024-331903>).

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Received 11 January 2024
Accepted 24 May 2024



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To cite: Chen A, Zhang VX, Zhang Q, *et al.* *Gut* Epub ahead of print: [please include Day Month Year]. doi:10.1136/gutjnl-2024-331903

ABSTRACT

Objective Fat mass and obesity-associated protein (FTO), an eraser of *N*⁶-methyladenosine (m6A), plays oncogenic roles in various cancers. However, its role in hepatocellular carcinoma (HCC) is unclear. Furthermore, small extracellular vesicles (sEVs, or exosomes) are critical mediators of tumourigenesis and metastasis, but the relationship between FTO-mediated m6A modification and sEVs in HCC is unknown.

Design The functions and mechanisms of FTO and glycoprotein non-metastatic melanoma protein B (GNPMB) in HCC progression were investigated in vitro and in vivo. Neutralising antibody of syndecan-4 (SDC4) was used to assess the significance of sEV-GNPMB. FTO inhibitor CS2 was used to examine the effects on anti-PD-1 and sorafenib treatment.

Results FTO expression was upregulated in patient HCC tumours. Functionally, FTO promoted HCC cell proliferation, migration and invasion in vitro, and tumour growth and metastasis in vivo. FTO knockdown enhanced the activation and recruitment of tumour-infiltrating CD8⁺ T cells. Furthermore, we identified GNPMB to be a downstream target of FTO, which reduced the m6A abundance of GNPMB, hence, stabilising it from degradation by YTH *N*⁶-methyladenosine RNA binding protein F2. Of note, GNPMB was packaged into sEVs derived from HCC cells and bound to the surface receptor SDC4 of CD8⁺ T cells, resulting in the inhibition of CD8⁺ T cell activation. A potential FTO inhibitor, CS2, suppresses the oncogenic functions of HCC cells and enhances the sensitivity of anti-PD-1 and sorafenib treatment.

Conclusion Targeting the FTO/m6A/GNPMB axis could significantly suppress tumour growth and metastasis, and enhance immune activation, highlighting the potential of targeting FTO signalling with effective inhibitors for HCC therapy.

INTRODUCTION

The *N*⁶-methyladenosine (m6A) modification is the most prevalent chemical modification in eukaryotic mRNA.^{1,2} m6A and its regulatory enzymes and reader proteins play critical roles in post-transcriptional gene regulation,^{3–6} and their dysregulation has been associated with various diseases, especially cancer.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Fat mass and obesity-associated protein (FTO), the first identified RNA demethylase, plays a pivotal role in regulating *N*⁶-methyladenosine (m6A) modification.
- ⇒ FTO plays an oncogenic or tumour-suppressive role in different cancers.
- ⇒ FTO inhibition has been reported to sensitise leukaemic cells to T cell cytotoxicity and overcome hypomethylating agent-induced immune evasion.
- ⇒ Glycoprotein non-metastatic melanoma protein B (GNPMB) increases the phosphorylation of ERK, AKT and WNT in different models and cell types.
- ⇒ Syndecan-4 (SDC4) is a receptor protein of CD8⁺ T cells.

WHAT THIS STUDY ADDS

- ⇒ FTO is pro-oncogenic in hepatocellular carcinoma (HCC).
- ⇒ FTO inhibited the activation and recruitment of tumour-infiltrating CD8⁺ T cells.
- ⇒ GNPMB was identified as a novel downstream target of FTO-mediated m6A modification.
- ⇒ GNPMB was packaged into exosomes derived from HCC cells and inhibited CD8⁺ T cell activation through binding to the surface receptor SDC4 of CD8⁺ T cells, resulting in cancer immune evasion.
- ⇒ FTO-GNPMB axis inhibited T cell activation by a cascade of m6A demethylation, small extracellular vesicles/exosomes and immunosuppression.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Targeting FTO may provide a new therapeutic approach for treating HCC.
- ⇒ Targeting FTO may suppress tumour growth and enhance the therapeutic effects of immune checkpoint blockade with anti-PD-1 and sorafenib in HCC.

The first identified RNA demethylase, fat mass and obesity-associated protein (FTO), plays a pivotal role in regulating m6A modification⁷ and has been

reported to play an oncogenic or tumour-suppressive role in multiple cancers.⁸ FTO inhibition has been reported to sensitise leukaemic cells to T cell cytotoxicity and overcome hypomethylating agent-induced immune evasion.⁹ Better understanding of the role and underlying molecular mechanisms of FTO in cancers will enhance the development of effective targeted therapeutics against FTO.

Hepatocellular carcinoma (HCC) is a prevalent malignancy worldwide. The role of FTO in HCC is controversial. It has been reported that in HCC, a high level of FTO promoted tumour growth through triggering the demethylation of pyruvate kinase (PKM2) mRNA and accelerating the translation.¹⁰ However, it has also been reported that FTO mRNA and protein levels were significantly downregulated in HCC tumours.¹¹

In this study, we found that FTO promoted HCC tumourigenesis and metastasis. Mechanistically, we identified glycoprotein non-metastatic melanoma protein B (GPNMB) as a downstream target of FTO-mediated m6A modification and revealed that GPNMB was packaged into small extracellular vesicles (sEVs) derived from HCC cells. The GPNMB on the sEVs inhibited CD8⁺ T cell activation through binding to the surface receptor syndecan-4 (SDC4) of CD8⁺ T cells, resulting in cancer immune evasion. Furthermore, FTO inhibitor, CS2 (NSC368390, also named brequinar), suppressed its oncogenic functions and sensitised HCC cells to immune checkpoint inhibitor anti-PD-1 treatment and enhancing tumour infiltrating CD8⁺ T cells. The results highlight a new therapeutic approach for HCC and some other cancers by targeting this pathway and sensitising cancer cells to existing immunotherapy and molecular targeted drug treatments.

MATERIALS AND METHODS

A total of 95 patients with primary HCC from The University of Hong Kong-Queen Mary Hospital (HKU-QMH) were included in this study. The details of the materials and methods used in this study including the cell culture techniques, gene manipulation in HCC cell lines, in vitro functional assays, and inhibitor treatments, in vivo animal studies, real-time quantitative PCR (qRT-PCR), next-generation sequencings and statistical analysis are included in online supplemental materials.

RESULTS

Frequent upregulation of FTO RNA demethylase in human HCC and other cancers

According to The Cancer Genome Atlas (TCGA) database, FTO expression was found to be significantly upregulated in seven different solid tumours, including HCC, cholangiocarcinoma, colonic adenocarcinoma, oesophageal squamous carcinoma, head and neck squamous carcinoma, kidney renal clear cell carcinoma and stomach adenocarcinoma (online supplemental figure S1A). This upregulation was confirmed in our in-house HKU-QMH RNA-sequencing (RNA-seq) database with 41 pairs of human HBV-associated HCC (online supplemental figure S1B) and corresponding non-tumour (NT) tissues (figure 1A, left panel) as well as in the TCGA database with 50 paired HCC patient samples (figure 1A, right panel). Additionally, FTO upregulation was verified using qRT-PCR in our expanded HCC cohort consisting of 95 pairs of mainly HBV-associated primary HCC (online supplemental figure S1B), with 40% (n=38/95) showing a \geq twofold upregulation (figure 1B). Immunohistochemistry also revealed higher FTO protein levels in HCC tissues compared with NT tissues (figure 1C). Patients with high FTO expression (twofold cut-off) were found to

be significantly associated with poor overall and disease-free survival rates (figure 1D). FTO overexpression was also positively associated with more advanced tumour staging (figure 1E and online supplemental table 1). These findings indicate that FTO is upregulated in HCC and its upregulation is associated with more aggressive tumour and poorer prognosis.

Knockdown of FTO inhibited HCC progression and cell stemness properties

In our panel of human HCC cell lines, FTO was found to be highly expressed in HCC cell lines (MHCC97L (97L; authenticated to have no contamination; see online supplemental figure S1C) and HepG2) as compared with an immortalised normal liver cell line, while the lowest expression was observed in PLC/PRF/5 (PLC) cell line (online supplemental figure S1D). Using two independent short hairpin RNA (shRNA) sequences (shFTO#1 and #2), FTO stable knockdown (KD) was established in 97L and HepG2 cells (figure 2A, online supplemental figure S1E).

FTO KD significantly suppressed HCC cell proliferation (figure 2B), migration and invasion (figure 2C). To investigate whether silencing FTO could suppress tumour growth and progression of HCC cells in vivo, we employed an orthotopic mouse xenograft model. Luciferase-labelled 97L cells with FTO KD or non-target control (NTC) were injected into the livers of nude mice (figure 2D). A significant reduction in bioluminescence signals (figure 2E,F) and tumour mass (figure 2G) was observed on FTO KD. Additionally, a lower lung metastasis rate was detected in the FTO KD group (figure 2H). These findings demonstrate that FTO plays a crucial role in HCC cell proliferation, migration and invasion in vitro and tumour growth and progression in vivo. In addition, the effects of FTO KD on tumourigenicity were examined. FTO KD could significantly inhibit colony formation and sphere formation (self-renewal ability in vitro) in HCC cells (figure 2I,J). Moreover, FTO KD substantially downregulated the expression of cancer stemness genes, in these HCC cells (online supplemental figure S1F). A limiting dilution assay to assess tumour initiation ability was performed by subcutaneously injecting various numbers of 97L cells into nude mice (figure 2K). The results showed a significantly reduced incidence rate of tumour initiation on FTO KD ($p < 0.001$) (figure 2L,M). The suppressive effect on tumour formation was more evident for sequence shFTO#1, especially when lower numbers of HCC cells were injected. These findings revealed that FTO KD not only impedes tumour initiation but also suppresses tumour growth and progression in HCC.

KD of FTO inhibited tumour growth and enhanced infiltration, activation and recruitment of tumour-infiltrating CD8⁺ T cells

We queried whether the tumour-suppressive effects of FTO KD observed in immunodeficient mice were also present in immunocompetent mice. To this end, we used an orthotopic liver injection model and injected luciferase-labelled mouse hepatoma cells Hepa1-6 into immunocompetent C57BL/6 mice (figure 3A). On FTO KD, a significant reduction in bioluminescence signals (figure 3B) and tumour mass (figure 3C) was observed. Importantly, there were significantly increased numbers of tumour-infiltrating immune cells (CD45⁺) and cytotoxic CD8⁺ T cells (CD45⁺ CD8⁺) in the tumours with FTO KD. This was accompanied by a significant reduction in PD-1⁺ exhausted CD8⁺ T cells (figure 3D). Although the total tumour-infiltrating macrophages (CD45⁺ F4/80⁺) remained unchanged on FTO KD (online supplemental figure S2A), there was a trend of an increase in

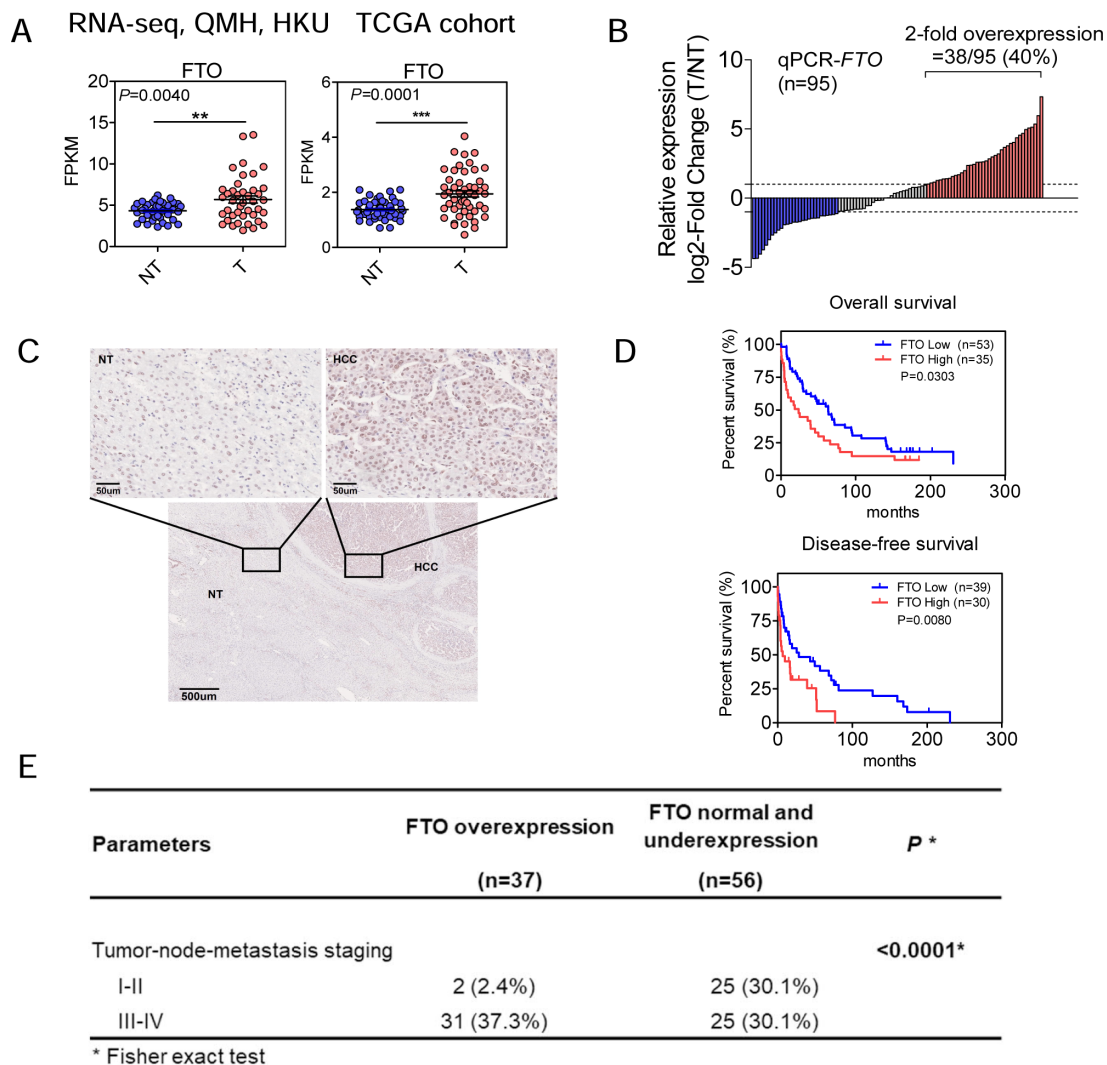


Figure 1 Frequent upregulation of FTO RNA demethylase in human HCC. (A) The RNA-seq data demonstrates that FTO is frequently upregulated in our in-house HKU-QMH HCC cohort (n=41) and TCGA paired HCC cohort (n=50). (B) The mRNA level of FTO was highly expressed in 40% of 95 pairs of patients' HCC samples when compared with non-tumourous liver tissues. (C) Representative immunohistochemical images of FTO in HCC tissue and non-tumourous liver tissue. (D) The high FTO expression was associated with a poorer overall survival of patients with HCC. (E) With a cut-off of twofold upregulation by qPCR, overexpression of FTO was significantly associated with more advanced tumour stages (p=0.00029). t-test, mean±SD, *p<0.05, **p<0.01, ***p<0.001. FTO, fat mass and obesity-associated protein; HCC, hepatocellular carcinoma; HKU, University of Hong Kong; QMH, Queen Mary Hospital; qPCR, quantitative PCR; NT, non-tumour; TCGA, The Cancer Genome Atlas.

antitumoural M1 macrophages (CD86⁺) (online supplemental figure S2B), but not pro-tumoural M2 macrophages (CD163⁺) (online supplemental figure S2C). These findings suggest that FTO KD enhances the infiltration of cytotoxic T cells and antitumoural macrophages to inhibit HCC tumour growth in immunocompetent mice.

To further investigate the effect of FTO KD on T cell activation, CD8⁺ T cells were isolated from the peripheral blood mononuclear cells (PBMCs) of healthy donors and cocultured with human HCC cell line MHCC97L with or without FTO KD. There was a significant increase in CD44⁺CD62L⁻ effector memory T cells on FTO silencing, as compared with the NTC group (figure 3E). FTO KD also enhanced the proliferation of human CD8⁺ T cells, as demonstrated with carboxyfluorescein succinimidyl ester staining, followed by gating the counts of proliferated cells using FlowJo software and normalised to the NTC group (online supplemental figure S2D). This indicates

that FTO may suppress T cell activation and proliferation in the HCC tumour microenvironment.

To strengthen this observation, we used the human monocyte cell line THP-1 and induced the cells to M0 differentiation and further to M1 or M2 polarisation.¹² M1 or M2 macrophages were then cocultured with HCC cells with or without FTO depletion. A significant promotion in the migratory ability of M1 macrophages was observed when they were cocultured with FTO KD 97L cells, while a reduction was seen with M2 macrophages on such coculture (figure 3F,G). Consistently, there was an increase in the expression of M1 markers, including CD68, CD80 and CD86, while decreased expression of CD204, an M2 marker, was seen with one of the FTO KD sequences (online supplemental figure S2E). These findings suggest that FTO KD promotes the activation and proliferation of cytotoxic T cells and enhances the migratory ability of antitumoural M1 macrophages in the HCC tumour microenvironment.

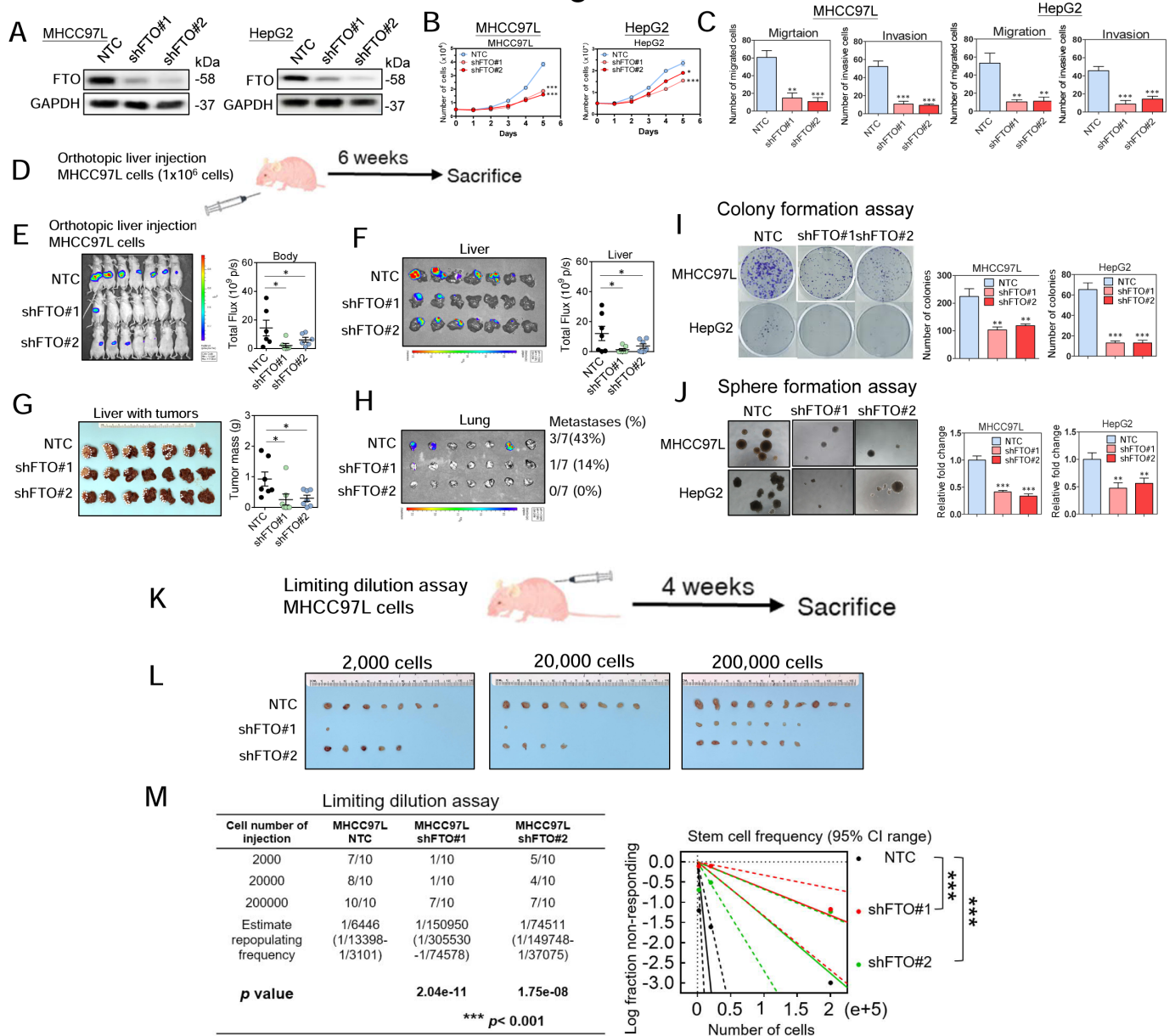


Figure 2 Knockdown (KD) of fat mass and obesity-associated protein (FTO) suppressed hepatocellular carcinoma cell proliferation, metastasis and self-renewal ability. (A) Western blotting showed successful stable KD of FTO in 97L and HepG2 cells. (B) Significant inhibition on cell proliferation on FTO KD. (C) The number of migrated or invaded cells was significantly reduced on FTO KD (n=3). (D) A schematic representation of the orthotopic injection model. (E) Bioluminescent images of nude mice subjected to orthotopic liver injection of 97L-luc cells (non-target control (NTC), shFTO#1 and shFTO#2) (n=7 for each group). (F) Bioluminescent images of livers and quantification of their bioluminescent intensities. (G) Dissected livers with tumours from the three groups of mice and weights of the dissected tumours. (H) Bioluminescent images of lung tissues and metastasis rate to lungs. (I) Colony formation assay and (J) sphere formation assay on FTO KD and NTC groups in 97L and HepG2 cells (n=3). (K) In vivo limiting dilution assay showing rates of tumour formation from subcutaneous injection of 2×10^3 , 2×10^4 and 2×10^5 97L cells. (L), (M) The tumour incidence rate for each group was recorded at the end of the experiments after 4 weeks. Tumour initiating capacity was analysed by the CIs with the formula of $CI=1/(\text{stem cell frequency})$. t-test, mean±SD, *p<0.05, **p<0.01, ***p<0.001.

RNA-seq and m6A-sequencing identified GPNMB as a downstream target of FTO-mediated m6A modification

To determine the molecular mechanism of FTO in HCC, RNA-seq was performed in FTO KD HCC cells. There were 60 upregulated genes and 133 downregulated genes when compared with NTC by two independent shRNAs in both HepG2 and 97L cells (figure 4A, online supplemental figure S3A). GO biological processes annotation analysis revealed that the upregulated and downregulated differentially expressed genes were significantly enriched in gene sets. Subsequently, we performed gene

expression correlation analysis between these targets and FTO in TCGA HCC cohorts. We could not identify gene targets among the upregulated genes having a negative correlation with FTO expression (data not shown). We then focused on the downregulated genes, particularly those included in positive regulation of cellular component movement processes, in which the gene count of this process was highest in the downregulated genes (figure 4B). Moreover, this pathway was relevant as we found that FTO KD mediated attenuation of cell migration (online supplemental figure S3B). The correlation between FTO and the

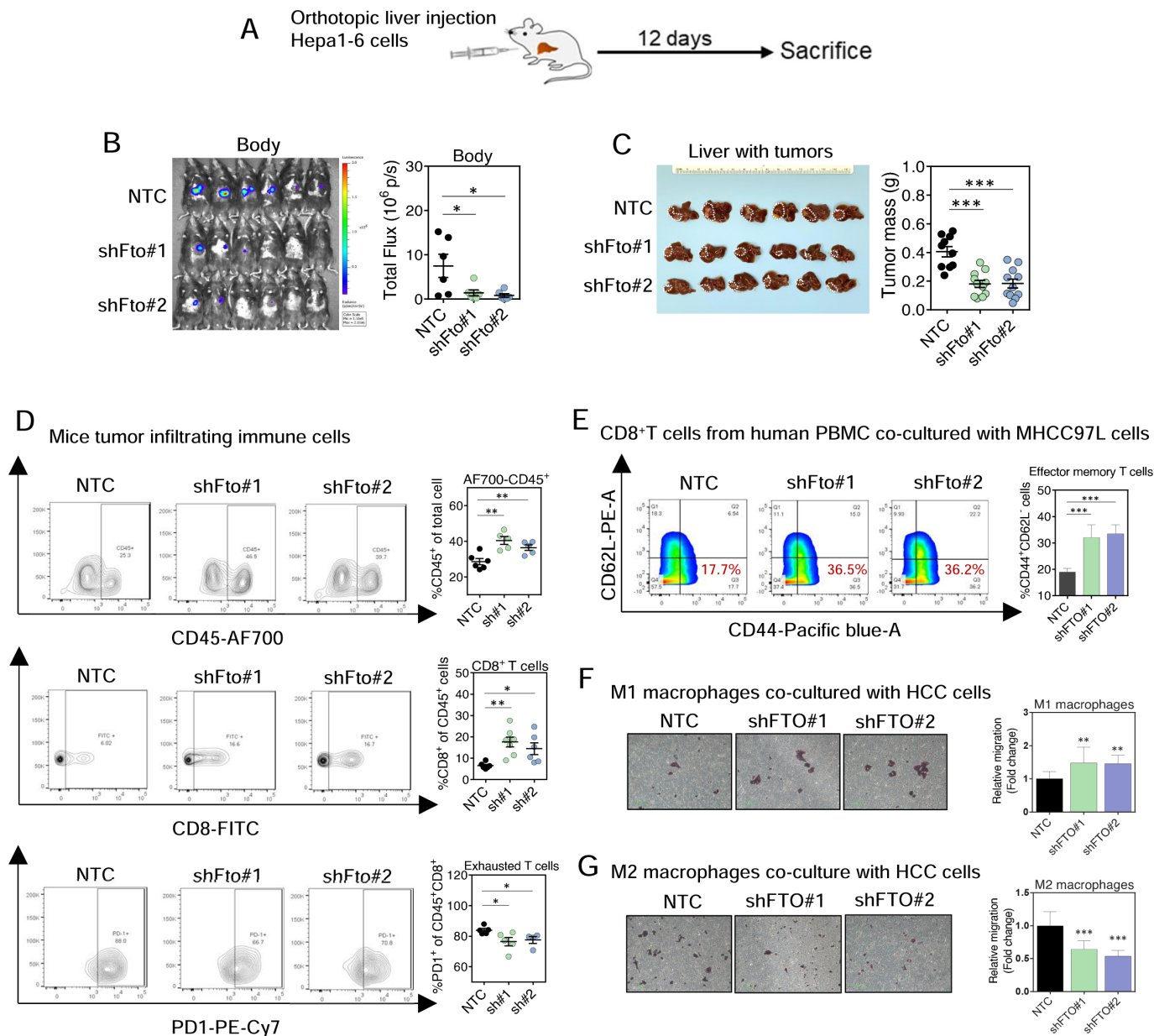


Figure 3 Fat mass and obesity-associated protein (FTO) knockdown (KD) enhanced immune response in vivo and in vitro. (A) Diagram showing orthotopic injection of luciferase-labelled Hepa1-6 cells (non-target control (NTC), shFto#1 and shFto#2) in C57BL/6 mice. (B) Bioluminescent images and quantification of their bioluminescent intensities. (n=6 in each group) (C) Livers with tumours and tumour mass. (D) Proportions of tumour infiltrating immune cells, CD45⁺, CD45⁺CD8⁺ and CD45⁺CD8⁺PD1⁺ cells, by flow cytometry. (E) Flow cytometry showing significant increase in CD44⁺CD62L⁻ effector memory CD8⁺T cells in human peripheral blood mononuclear cells (PBMCs) on coculturing with FTO KD hepatocellular carcinoma (HCC) cells. (F), (G) Migrated M1 and M2 macrophages after coculturing with HCC cells (NTC, shFto#1 and shFto#2). (E)-(G) Results were from three independent experiments. One-way analysis of variance followed by Dunnett comparison test, mean \pm SD, *p<0.05, **p<0.01, ***p<0.001.

potential target genes involved in the process were analysed, and the expression of these genes was also examined in the TCGA HCC cohort. Five potential downstream target genes of FTO were selected for further investigation. To this end, FTO KD significantly downregulated the expression of three potential downstream target genes (GPNMB, PDGFB and ROBO4) in both HepG2 (figure 4C) and 97L (figure 4D) cells.

To determine if the oncogenic effects of FTO depended on its m6A demethylase activity, we established stable FTO-wildtype (FTO-WT) and FTO-mutated (FTO-MUT)-overexpressing PLC cells (online supplemental figure S3C). The FTO-MUT had the mutation at the 316 site of the FTO protein amino acid sequence,

with replacement of arginine (R) by alanine (A), hence losing its demethylase activity.¹³ Stable overexpression of FTO-WT upregulated the mRNA levels of GPNMB in PLC cells as compared with the empty vector control, while this promoting effect was abolished on FTO mutation (figure 4E).

m6A-Seq was conducted on the mRNA samples isolated from FTO KD or NTC 97L cells to map the m6A modification. The data showed that the vast majority of m6A peaks were distributed in the coding sequence and 3' UTR regions (figure 4F). FTO KD induced an obvious increase of m6A peaks (figure 4G). The consensus motifs of m6A peaks were identified in the peak region (online supplemental figure S3D). An analysis of the m6A

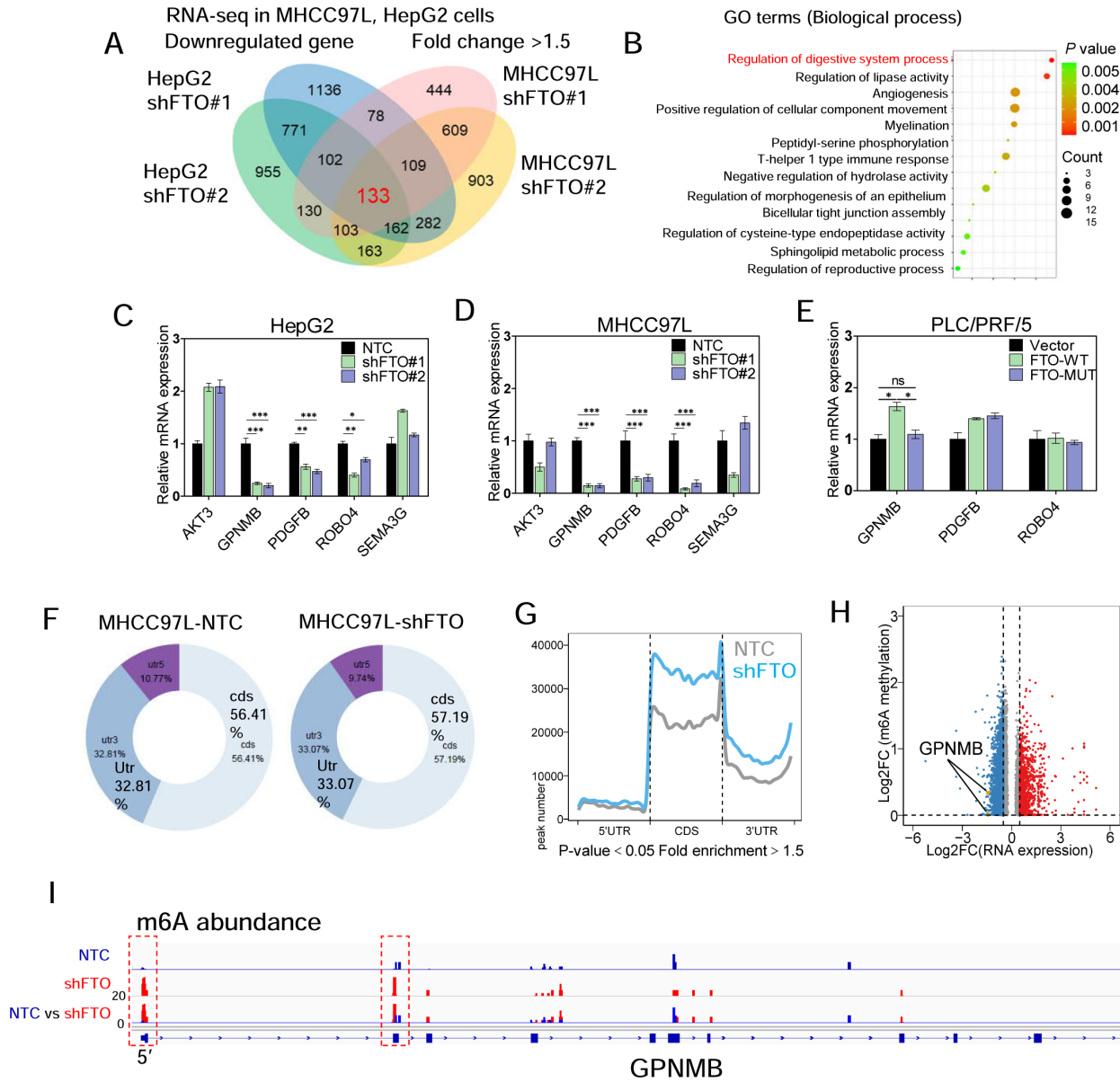


Figure 4 RNA-sequencing and m6A-methylated RNA immunoprecipitation assay identified glycoprotein non-metastatic melanoma protein B (GPNMB) as a downstream target of fat mass and obesity-associated protein (FTO)-mediated N^6 -methyladenosine (m6A) modification. (A) Venn diagram and (B) GO biological processes annotation analysis showing genes downregulated by >1.5-fold on FTO knockdown (KD) in HepG2 and 97L cells. (C and D) FTO KD significantly downregulated the mRNA level of potential downstream target genes (GPNMB, PDGFB and ROBO4) in 97L and HepG2 cells, respectively (n=3). (E) FTO- wildtype (FTO-WT) overexpression in PLC cells upregulated the mRNA levels of the three genes but this upregulation was abolished on FTO mutation (n=3) only for GPNMB. (F) Distribution of m6A peaks in different regions of mRNA as detected in m6A-seq assays conducted in control or FTO KD 97L cells. (G) The significantly increased (blue) m6A peaks on FTO KD in 97L cells. (H) The increased m6A abundance of mRNA in upregulated (red) or downregulated (blue) genes on FTO KD in 97L cells. (I) The m6A abundance in the GPNMB transcript. The m6A peaks were called by exome-Peak. t-test, mean \pm SD, * p <0.05, ** p <0.01, *** p <0.001. FTO-MUT, FTO mutated; NTC, non-target control.

modification on GPNMB mRNA on silencing FTO in 97L cells revealed an obvious increase in m6A abundance (figure 4H,I). This is in line with the aforementioned finding that FTO KD significantly decreased the mRNA level of GPNMB (figure 4D). In summary, GPNMB was identified as a direct target of m6A modification.

FTO KD reduced GPNMB mRNA stability via an m6A-YTH N^6 -methyladenosine RNA binding protein F2-dependent pathway
According to TCGA database, GPNMB is highly expressed in HCC tumours as well as a number of other cancers (online supplemental figure S4A). GPNMB was found to

be significantly upregulated in HCC tumours in both our in-house RNA-seq database with 41 pairs of HCC samples and the TCGA database with 375 HCC patients' samples (figure 5A, left and right panels). GPNMB mRNA expression also showed a positive correlation with FTO expression in TCGA HCC cohort (online supplemental figure S4B). Moreover, silencing FTO significantly downregulated the mRNA and protein levels of GPNMB in 97L cells (figure 5B). Strikingly, FTO KD resulted in global increase in m6A abundance in HCC cells (figure 5C). These findings suggest the link between FTO and GPNMB expression in HCC, with FTO

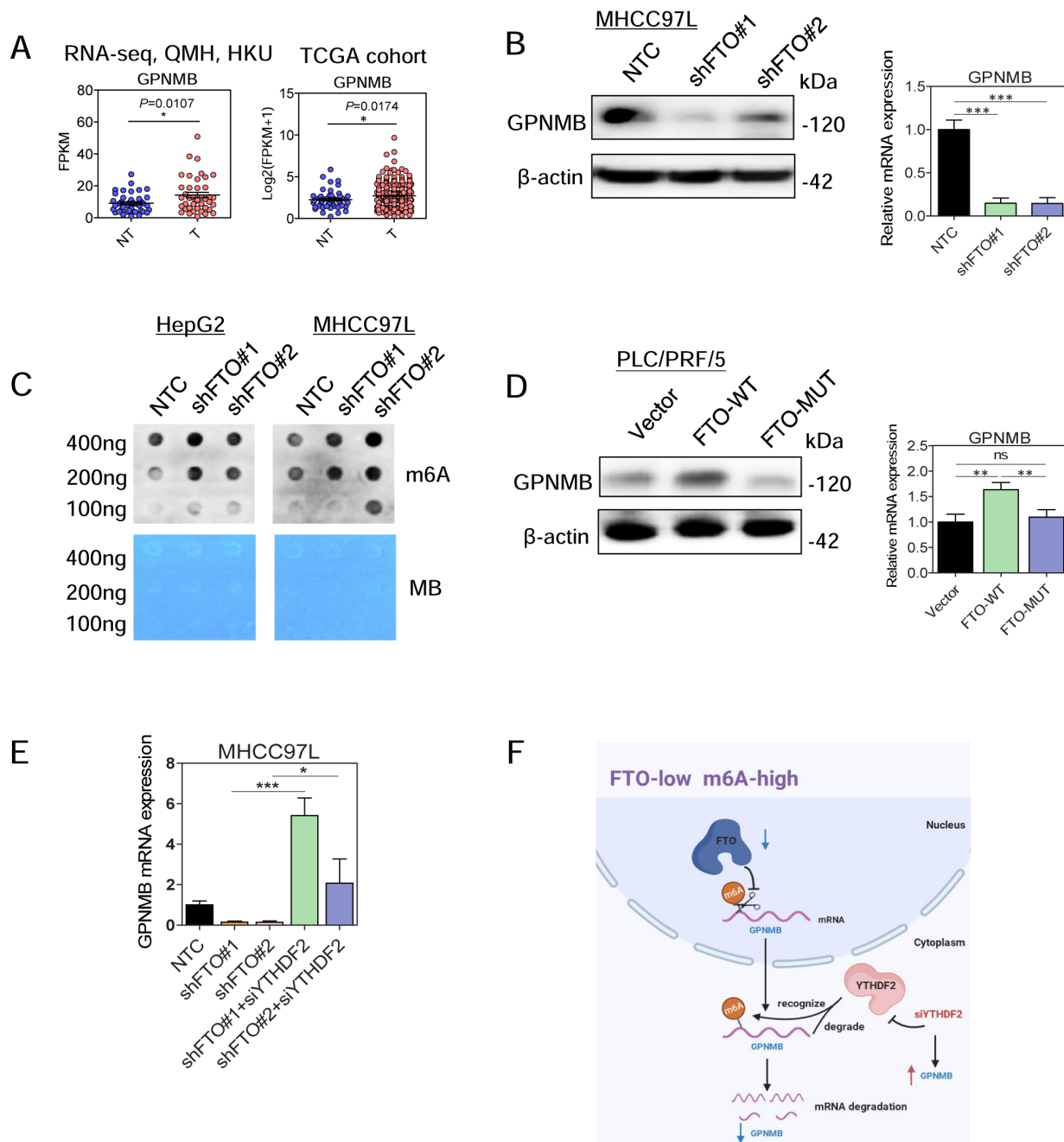


Figure 5 FTO KD attenuated GPNMB mRNA stability through YTHDF2. (A) GPNMB was frequently upregulated in our in-house RNA-seq HCC cohort (n=41 pairs) and TCGA HCC cohort (n=375). (B) The mRNA and protein levels of GPNMB were reduced in FTO stable KD 97L cells. (C) Determination of m6A abundance by Dot Blot assay in HepG2 and 97L cells on FTO stable KD. (D) The mRNA and protein level of GPNMB in PLC cells on overexpression of FTO-WT or FTO-MUT. (E) The mRNA levels of GPNMB in FTO KD 97L cells with or without silencing YTHDF2. (n=3). (F) A schematic diagram illustrating GPNMB expression was regulated by FTO-mediated m6A modification through YTHDF2-mediated mRNA degradation. t-test, mean \pm SD, * p <0.05, ** p <0.01, *** p <0.001). FTO, fat mass and obesity-associated protein; FTO-MUT, FTO mutated; FTO-WT, FTO-wildtype; GPNMP, glycoprotein non-metastatic melanoma protein B; HCC, hepatocellular carcinoma; HKU, University of Hong Kong; QMH, Queen Mary Hospital; qPCR, quantitative PCR; m6A, N^6 -methyladenosine; NTC, non-target control; NT, non-tumour; TCGA, The Cancer Genome Atlas; YTHDF2, YTH N^6 -methyladenosine RNA binding protein F2.

KD leading to downregulation of GPNMB expression and an increase in m6A abundance.

Next, we confirmed that FTO expression affected GPNMB expression through an m6A-dependent mechanism. To this end, overexpression of FTO-WT increased the mRNA and protein levels of GPNMB as compared with the empty vector control in PLC cells, but this effect was abolished with expression of FTO-MUT (figure 5D). In the in vivo limiting dilution assay to test

tumour initiation ability, overexpression of FTO-WT increased the tumour incidence on injection of both 2×10^3 and 2×10^4 HCC cells. In addition, the tumour size was larger in the FTO-WT group with the injection of 2×10^5 cells (online supplemental figure S4C). For the FTO-MUT group, the tumour incidence was reduced with the injection of 2×10^3 cells and unchanged with the injection of 2×10^4 and 2×10^5 cells. The estimated tumour initiation frequency showed a significant increase in

the FTO-WT group when compared with the FTO-MUT group ($p < 0.001$) (online supplemental figure S4D), suggesting that the tumorigenesis-promoting properties of FTO likely depend on its m6A demethylase activity.

Previous studies had shown that YTH N^6 -methyladenosine RNA binding protein F2 (YTHDF2) selectively binds to m6A-containing mRNA and degrades it.¹⁴ To investigate this, we knocked down YTHDF2 in 97L cells using small interfering RNA specifically targeting YTHDF2 (online supplemental figure S4E). YTHDF2 KD remarkably rescued the downregulation of GPNMB mRNA induced by silencing FTO (figure 5E). The finding suggests that the potential downstream target of FTO, GPNMB, has m6A modification by FTO but is also subjected to YTHDF2-dependent mRNA degradation. This adds another layer of complexity to the regulation of GPNMB expression and highlights the interplay between FTO and YTHDF2 in controlling the stability of GPNMB in HCC (figure 5F).

GPNMB KD inhibited the pro-oncogenic phenotype of HCC

To investigate the role of GPNMB as a potential downstream target of FTO in HCC, stable KD of GPNMB was established in 97L and HepG2 cells using two independent shRNA sequences. Successful KD at the protein level was confirmed in both cell lines (online supplemental figure S5A,B). Silencing GPNMB significantly inhibited cell proliferation (figure 6A) and migration (figure 6B) in both cells. We also assessed the tumour growth in vivo using an orthotopic injection model with luciferase-labelled 97L cells with or without GPNMB KD. GPNMB KD exerted a significant reduction in the tumour-forming ability in the liver (online supplemental figure S5C,D; figure 6C) and resulted in a lower incidence of lung metastasis (figure 6D). These findings indicate that GPNMB plays a crucial role in promoting HCC tumour growth and metastasis, further supporting its role as a downstream target of FTO in HCC. Furthermore, GPNMB KD significantly suppressed the colony formation and sphere formation abilities in 97L and HepG2 cells (figure 6E,F). To further assess the tumour-initiating ability of GPNMB KD cells in vivo, a limiting dilution assay, in which varying numbers of HCC cells (2×10^5 , 2×10^4 and 2×10^3) were subcutaneously injected into nude mice, showed a significantly lower tumour-initiating capacity in shGPNMB cells compared with NTC cells (figure 6G). Extreme limiting dilution analysis on the limiting dilution assay showed a 3.6-fold reduction in 'stem-cell frequency' ($p = 8.94 \times 10^{-4}$), which estimates the frequency of self-renewing tumour-initiating cells (figure 6H). Taken together, these results confirm the functional roles of GPNMB in promoting HCC tumour initiation, growth and metastasis.

GPNMB activated AKT, ERK and WNT pathways to promote HCC progression

To further investigate the underlying mechanism of GPNMB in HCC progression, we overexpressed GPNMB in FTO KD 97L cells (online supplemental figure S6A). GPNMB overexpression significantly rescued the suppressed abilities of cell proliferation (figure 7A), migration (figure 7B, online supplemental figure S6B), sphere formation (figure 7C, online supplemental figure S6C) in FTO KD 97L cells. Previous studies have shown that GPNMB signalling increases the phosphorylation of ERK and AKT in different models and cell types,^{15–17} as well as activates the canonical WNT pathway, as evidenced by increased β -catenin transcriptional activity in breast cancer cells.^{18,19} Therefore, we examined the activation of WNT, AKT and ERK pathways. GPNMB KD downregulated the expression of β -catenin,

phosphorylated AKT and phosphorylated ERK in 97L cells (figure 7D, online supplemental figure S6D), while GPNMB overexpression in the FTO KD 97L cells abrogated these effects (figure 7E, online supplemental figure S6E). These findings suggest that GPNMB may promote HCC progression through the activation of WNT, AKT and ERK signalling pathways.

GPNMB was packaged into sEVs derived from HCC cells and suppressed immune cell activation

Previous studies showed that GPNMB could be packaged into sEVs of mouse melanoma cells.²⁰ In this study, we showed that GPNMB was also packaged into sEVs produced by HCC cells (figure 7F; online supplemental figure S6G). Of note, we found presence and also upregulation of GPNMB in sEVs of HCC patients' plasma when compared with those from healthy subjects (online supplemental figure S6F). Next, we extracted sEVs from FTO-overexpressing (FTO-WT or FTO-MUT (R316A)) PLC and 97L cells with GPNMB KD. We found that sEVs derived from the FTO-WT-overexpressing HCC cells contained high levels of GPNMB protein, whereas those from the FTO-MUT-overexpressing HCC cells had GPNMB protein levels only comparable to the control group (figure 7F and online supplemental figure S6G). Furthermore, sEVs derived from GPNMB KD HCC cells showed a much lower level of GPNMB protein compared with the NTC group (figure 7F and online supplemental figure S6G). Additionally, nanoparticle tracking analysis identified the sEVs derived from HCC cell lines had a size peak around 120–140 nm (online supplemental figure S6H). Transmission electron microscopy confirmed that the sEVs had a cup-shaped morphology and a diameter of around 30–160 nm. We next examined whether GPNMB was localised within sEVs or present on the surface of sEVs. First, our immunogold labelling indicated that GPNMB was present on the membrane of sEVs (figure 7G). Then, we treated sEVs from 97L with proteinase K to degrade proteins at a concentration that led to the degradation of the outer membrane proteins such as CD63, but not the intravesicular HSP70 (figure 7H). The findings showed that the distribution of GPNMB was limited to the sEV surface. These findings suggest that GPNMB is present on the surface of sEVs derived from HCC cells.

sEV-GPNMB from HCC cells bound to SDC4 on CD8⁺ T cells and suppressed activation of CD8⁺ T cells

We questioned how FTO regulated GPNMB to affect immune microenvironment. Our previous single-cell RNA sequencing study on HCC patients' tumours¹² revealed that the expression of GPNMB negatively correlated with the fraction of CD8⁺ T cells ($p = 0.015$, $r = -0.85$) (figure 7I). To address the relationship of GPNMB, sEVs and CD8⁺ T cells, HCC-cell derived sEVs were added to CD8⁺ T cells isolated from PBMC of healthy subjects. A significant reduction of CD44⁺CD62L⁻ effector memory T cell proportion was observed in sEVs-NTC treated CD8⁺ T cells, as compared with PBS control (figure 7J). In contrast, this suppressive effect on CD8⁺ T cells was abrogated by sEVs from shGPNMB HCC cells (figure 7J and online supplemental figure S6I). Our finding suggests that GPNMB may inhibit the activation of CD8⁺ T cells via its packaging into sEVs.

Next, we examined whether GPNMB located on the surface of sEVs could bind to SDC4, which is a receptor protein of CD8⁺ T cells. To this end, we overexpressed a fusion protein, GFP-GPNMB, in 97L cells, and used the sEVs derived from GFP-GPNMB-overexpressing cells to treat CD8⁺ T cells ($n = 4 \times 10^5$) isolated from the PBMCs of healthy subjects at a

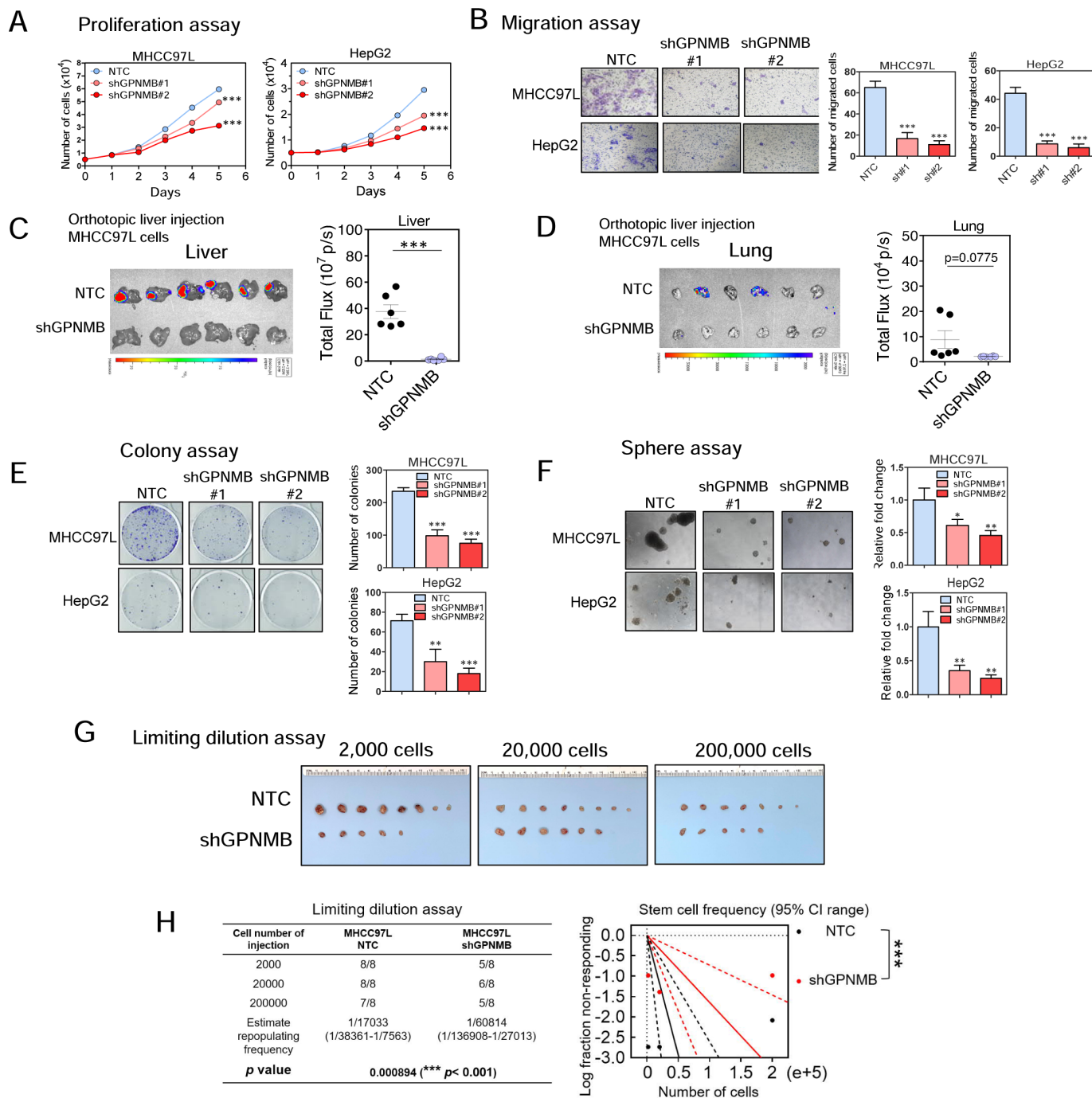


Figure 6 Glycoprotein non-metastatic melanoma protein B (GPNMB) knockdown (KD) inhibited hepatocellular carcinoma tumour growth metastasis and stemness. (A) GPNMB KD significantly inhibited proliferation of HepG2 and 97L cells. (B) The numbers of migrated cells were significantly reduced on fat mass and obesity-associated protein KD ($n=3$). (C and D) Orthotopic liver injection with Hepa1-6 with or without GPNMB KD in nude mice. Bioluminescence images of livers and lungs, with quantification of their bioluminescent intensities. (E) KD of GPNMB significantly suppressed the colony formation ability in 97L and HepG2 cells ($n=3$). (F) KD of GPNMB significantly reduced the size and number of hepatospheres formed by 97L and HepG2 cells ($n=3$). (G and H) Limiting dilution assay to assess the effects of GPNMB KD on tumourigenicity in nude mice. 2×10^3 , 2×10^4 and 2×10^5 97L cells were injected subcutaneously into nude mice. The tumour incidence rate for each group was recorded at the end of the experiments. Tumour initiating capacity was calculated by the CIs with the formula of $CI=1/(\text{stem cell frequency})$. t-test, mean \pm SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NTC, non-target control.

final concentration of 20 $\mu\text{g/mL}$ for 16 hours. SDC4 expression was detected in the protein complexes pulled down by the GFP antibody (figure 7K).

To investigate whether GPNMB located on the surface of sEVs inhibited CD8^+ T cell activation through binding to SDC4, we pre-treated CD8^+ T cells with a mouse IgG

antibody or neutralising antibody (NA)^{21 22} against SDC4 ectodomain for 2 hours, followed by treatment with the sEVs derived from 97L-NTC cells, the NA of SDC4 significantly enhanced the activation of CD8^+ T cells as compared with the IgG control group (figure 7L and online supplemental figure S6J). Taken altogether, the results showed

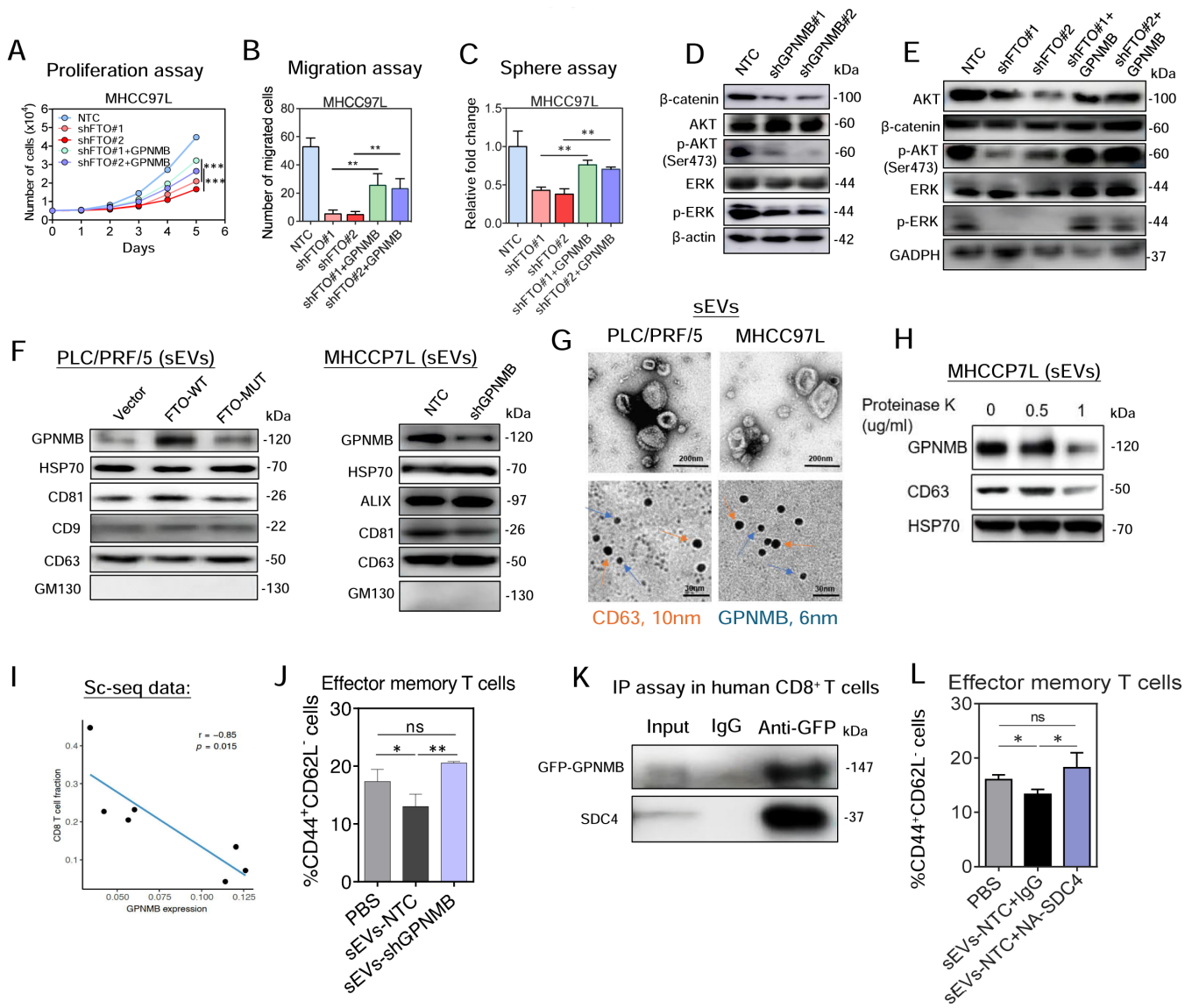


Figure 7 Fat mass and obesity-associated protein (FTO) activated AKT, ERK and WNT pathways and suppressed immune cell activation through upregulating glycoprotein non-metastatic melanoma protein B (GPNMB). (A) The cell proliferation, (B) migratory ability and (C) sphere forming ability on rescue of FTO knock down (KD) with overexpression of GPNMB in 97 L cells (n=3). (D) Western blotting showing GPNMB KD suppressed activation of AKT, ERK, WNT signalling pathway in 97L cells. (E) Overexpression of GPNMB rescued the activation of AKT, ERK, WNT signalling pathways shown with western blotting in 97L cells. (F) Western blots showing presence of GPNMB in small extracellular vesicles (sEVs) derived from PLC (EV, FTO-wildtype (FTO-WT), FTO-mutated (FTO-MUT)) and 97L cells (non-target control (NTC), shGPNMB). CD63, CD81, HSP70, CD9, CD63 and ALIX are positive EV markers and GM130 is a negative EV marker, and they were used as loading control. (G) Representative electron micrographs showing the morphology of sEVs from PLC and 97L cells (upper panel) (scale bar=200 nm), and immunogold labelling (lower panel) (scale bar=30 nm). (H) Western blot showing the protein expression of GPNMB, surface-sEVs marker CD63 and intra-sEV marker HSP70 in 97L-derived sEVs treated with increasing concentrations of proteinase K. (I) The correlation between GPNMB expression and CD8⁺ T cell fraction was confirmed in Sc-seq data of hepatocellular carcinoma patients. (J) Flow cytometry showing significant increase CD44⁺CD62L⁻ effector memory T cells on treating with sEVs derived from GPNMB KD 97L cells compared with NTC group. (K) Co-immunoprecipitation assay showing GFP-GPNMB located on the surface of sEVs interacted with SDC4 as a surface receptor on human CD8⁺ T cells. Total cell lysate (input) was used as positive control. (L) Flow cytometry showing significant increase CD44⁺CD62L⁻ effector memory T cells on treating with SDC4 blocking antibody. t-test, mean±SD, **p<0.01, ***p<0.001.

that GPNMB inhibited CD8⁺ T cell activation by directly binding to SDC4 via sEVs.

Blockade of FTO sensitised HCC to anti-PD-1 immune checkpoint blockade therapy

We employed three mouse models to examine the effects of targeting FTO. In an orthotopic liver injection mouse model, pharmacological inhibition of FTO by its specific inhibitor CS2

suppressed tumour growth and enhanced the antitumour efficiency of tyrosine kinase inhibitor, Sorafenib, in combination treatment (online supplemental figure S7A,B). CS2 treatment significantly downregulated the protein expression of GPNMB, phosphorylated AKT and phosphorylated ERK (online supplemental figure S7C). On the other hand, CS2 alone or combined with anti-PD-1 also significantly increased the infiltration of CD45⁺ F4/80⁺ macrophages and antitumoural M1 macrophages

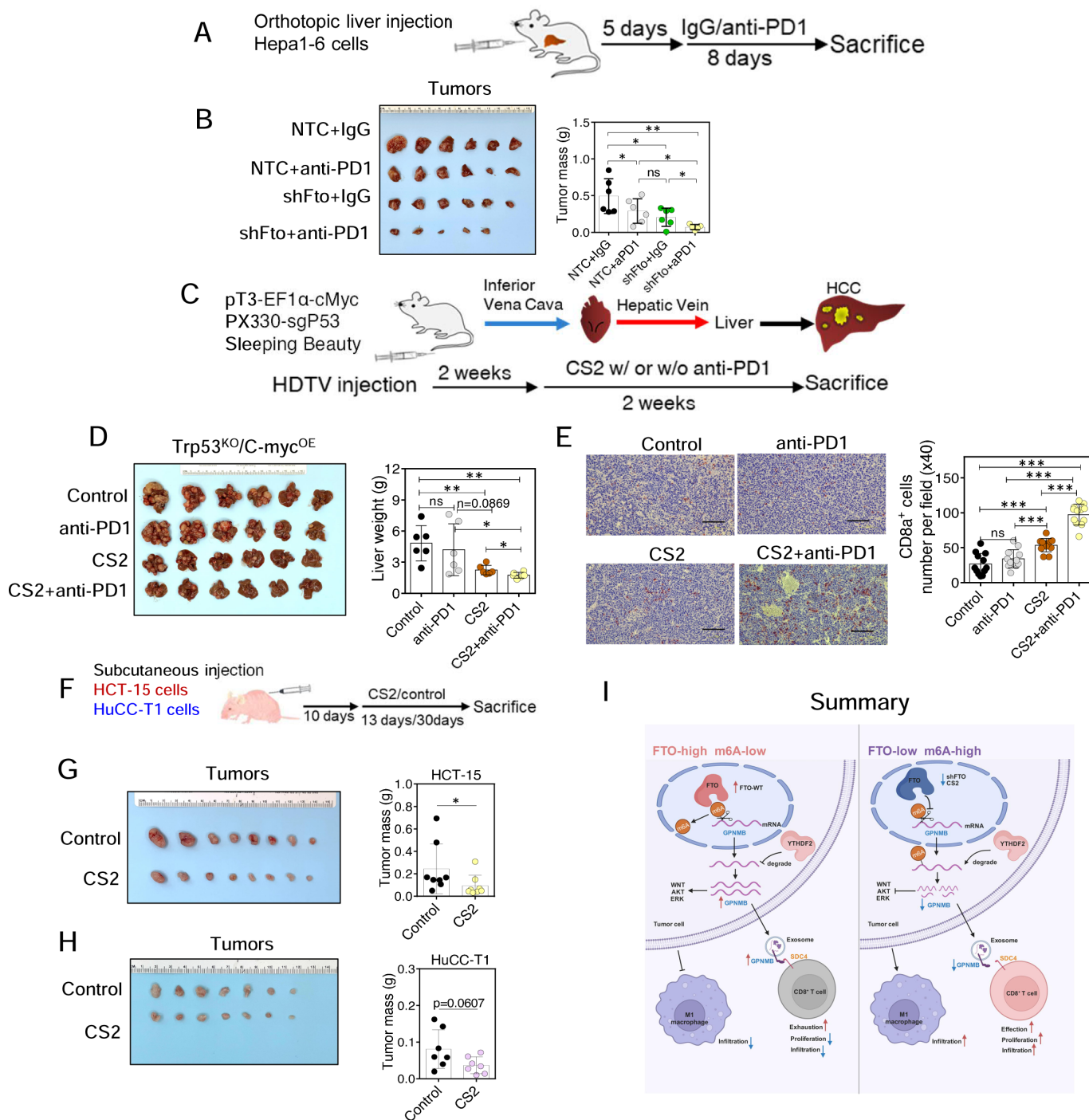


Figure 8 Blockade of fat mass and obesity-associated protein (FTO) sensitised hepatocellular carcinoma to anti-PD-1 immune checkpoint blockade therapy. (A) Schematic diagram illustrating the workflow of anti-PD-1 treatment in orthotopic liver injection mouse model with Hepa1-6 cells injection (non-target control (NTC)/shFto+IgG, NTC/shFto+anti-PD-1). (B) Tumours dissected from livers and their tumour masses. (C) A schematic summary of FTO inhibitor CS2 and anti-PD-1 treatment in hydrodynamic tail vein injection (p53 KO/c-Myc) model with induced tumours in mice. (D) Livers with tumours and liver weights representing the tumour masses on treatment with anti-PD-1, CS2 and CS2+anti-PD-1 combination treatment. (E) Immunohistochemical staining of tumour infiltrating CD8⁺ T cells in liver tumour tissues of mice (left panel) (scale bar=100 μ m) and quantification (right panel). (F) A summary of FTO inhibitor treatment in human colorectal adenocarcinoma HCT-15 cells and human cholangiocarcinoma HuCC-T1 cell-derived subcutaneous xenografts. (G and H) Tumour xenografts of HCT-15 and HuCC-T1 cells treated with vehicle control and CS2. (I) A schematic summary of this study. t-test, mean \pm SD, * p <0.05, ** p <0.01.

(CD45⁺ F4/80⁺ CD86⁺), when compared with control group (online supplemental figure S7D). Since silencing FTO expanded CD8⁺ T cell infiltration and effector memory CD8⁺ T cell activation (figure 3D,E), we evaluated the potential enhancing effect of targeting FTO using genetic silencing and immune

checkpoint blockade (ICB) with an immune checkpoint inhibitor anti-PD-1 in an orthotopic liver injection mouse model, in which mouse hepatoma Hepa1-6 cells with or without FTO KD were injected (figure 8A). The combination treatment of anti-PD-1 and FTO KD showed the most significant tumour growth

suppression (figure 8B). Finally, a hydrodynamic tail-vein injection (HDTVi) model was employed to induce spontaneous HCC tumours in C57BL/6 mice with CRISPR/Cas9-mediated loss-of-function genome editing of endogenous TP53 and Sleeping Beauty transposon-driven C-Myc overexpression (Trp53KO/C-MycOE) (figure 8C). Anti-PD-1 alone failed to impede tumour development, consistent with previous findings.²³ In contrast, targeting FTO pharmacologically with CS2 alone significantly decreased the tumour masses, and the combination of CS2-anti-PD-1 resulted in further significant reduction of tumour masses (figure 8D). These findings suggest that targeting FTO, either by pharmacological inhibition or genetic silencing, suppresses tumour growth and enhances the therapeutic effects of immune checkpoint blockade with anti-PD-1 and Sorafenib in HCC mouse models.

Immunohistochemistry on the HDTVi mouse tumour tissues revealed that the number of tumour-infiltrating CD8⁺ T cells was remarkably increased in the CS2-anti-PD-1 combination group compared with either the CS2 or anti-PD-1 single treatment group (figure 8E). Furthermore, depletion of CD8⁺ T cells using anti-CD8 antibody in immunocompetent mice abrogated the tumour-suppressive effect of CS2 and CS2/anti-PD-1 combo (online supplemental figure S8A–C). As CS2 is a specific inhibitor of FTO demethylase, these findings suggest that FTO/m6A promotes HCC progression through the activation of AKT and ERK signalling pathways.

Additionally, we tested the therapeutic efficiency of the FTO inhibitor CS2 in two other cancer cell lines with high FTO expression levels: HCT-15 (human colorectal adenocarcinoma) and HuCC-T1 (human cholangiocarcinoma), based on TCGA data (online supplemental figure S1A). In a subcutaneous xenograft mouse model, mice were treated with CS2 after tumour formation (figure 8F). CS2 was found to significantly decrease the tumour growth in HCT-15 cells (figure 8G) and a trend in HuCC-T1 cells (figure 8H). Taken together, the findings suggest that targeting FTO with CS2 may be a promising treatment strategy for HCC and possibly for some other cancer types. Moreover, it may potentially sensitise HCC to ICB therapy.

DISCUSSION

This study demonstrates the crucial role of FTO in promoting HCC growth, metastasis and stemness. We also identified GPNMB as a novel downstream target of FTO, which regulated the m6A demethylation of GPNMB mRNA. Importantly, we demonstrated that GPNMB was packaged into sEVs derived from HCC cells and bound to the surface receptor SDC4 of CD8⁺ T cells, resulting in the inhibition of CD8⁺ T cell activation (figure 8I).

In this study, our data provides important insights into the role of FTO in regulating the immune response in cancer. We demonstrated that FTO upregulated the protein level of GPNMB in sEVs through its m6A demethylase activity. Furthermore, GPNMB was present and also upregulated in HCC patients' plasma sEVs. Single-cell RNA sequencing study on human HCC tumours revealed that the expression of GPNMB negatively correlated with the fraction of CD8⁺ T cells. GPNMB on the surface of sEVs was found to inhibit CD8⁺ T cell activation by directly binding to SDC4, a receptor upregulated on activated T cells and known to enhance T cell activation.^{24,25} This GPNMB/SDC4 pathway has been previously reported to inhibit human allogeneic T-cell responses. Additionally, FTO KD was shown to activate immune response by sensitising melanoma cells to anti-PD-1 treatment in mice.²⁶ A recent study reported that

GPNMB showed immune suppression in hepatic parenchymal cells.²⁷ Our findings in this study not only showed the immune suppressive effect of the FTO/GPNMB axis, but also uncovered the novel finding that sEVs play an important role in mediating the immune suppressive effect by interaction with SDC4 in CD8⁺ T cells. The sEV-GPNMB may be as a plausible marker of HCC prognosis.

This study demonstrates the potential therapeutic value of CS2, an FTO inhibitor, in suppressing tumour growth and enhancing the effectiveness of existing cancer treatments. CS2 has previously been reported to inhibit dihydroorotate dehydrogenase, block de novo pyrimidine biosynthesis²⁸ and exhibit potent antileukaemic efficacy in samples with high FTO expression through binding directly to FTO protein.⁹ It has also been tested in clinical trials for cancer therapy.^{29–32} In this study, CS2 was found to suppress tumour growth and enhance the antitumour efficiency of Sorafenib in a mouse model. Furthermore, the combination of CS2 and anti-PD-1 treatment resulted in a significant reduction in tumour mass compared with single-agent treatment in spontaneous tumorigenesis model. Interestingly, CS2 also decreased tumour mass in human colorectal adenocarcinoma and cholangiocarcinoma cell lines with high FTO expression. Depletion of CD8⁺ T cells in immunocompetent mice abrogated the tumour-suppressing effect of CS2 and CS2/anti-PD-1 combo, further confirming the immune suppressive effect mediated via interaction with CD8⁺ T cells. While further systematic studies are needed to confirm the overall anticancer efficacy of CS2 and explore other potential mechanisms of action, these findings suggest that CS2 may have therapeutic potential in different types of cancers with high FTO expression.

In summary, our results demonstrated that targeting the FTO/m6A/GPNMB axis could significantly suppress tumour growth, metastasis, stemness and enhance immune activation, highlighting the broad potential of targeting FTO signalling by effective inhibitors (alone or in combination with other therapeutic agents) for cancer therapy. These insights contribute to the understanding of the molecular mechanisms underlying FTO's role in HCC and provide a foundation for the development of targeted therapeutics against FTO.

Acknowledgements The authors thank the Centre for PanorOmic Sciences Imaging and Flow Cytometry Core at the LKS Faculty of Medicine of The University of Hong Kong for technical assistance and support; the Centre for Comparative Medicine Research of The University of Hong Kong for providing animals, husbandry and facilities for animal experimentation; and the Electron Microscope Unit for providing technical support. Figure 5G and graphical abstract figure were created with Biorender.com.

Contributors AC, VXZ and IO-LN provided the study concept and design. AC, VXZ and IO-LN wrote the manuscript. AC, VXZ, QZ, DW-HH, XL, CMW and IO-LN interpreted and analysed the data. AC, VXZ, KM-FS, LT, HH, XW, EL, JL, MFJL performed the experiments. IO-LN and MFJL collected the patients' samples. LT and XW advised on extracellular vesicle-related experiments. IO-LN provided financial support. IO-LN is responsible for the overall content as the guarantor and supervised the project. All authors approved the final version of the manuscript.

Funding This project was supported in part by grants from the Research Grants Council of Hong Kong—Theme-based Research Scheme (T12-716/22R), Innovation and Technology Commission grant of Hong Kong SAR Government to State Key Laboratory of Liver Research (ITC PD/17-9), National Natural Science Foundation of China (82394451) and University Development Fund of The University of Hong Kong. IOLN is Loke Yew Professor in Pathology. AC is a Hong Kong Scholar.

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval All experimental procedures on mice were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of

Hong Kong (CULATR 5089-19 and 5688-21) and conducted in accordance with the Animals (Control of Experiments) Ordinance of Hong Kong.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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