Molecular and Cellular Biochemistry (2022) 477:1555–1568
https://doi.org/10.1007/s11010-022-04392-6

Menin regulates lipid deposition in mouse hepatocytes via interacting with transcription factor FoxO1

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Received: 31 July 2021 / Accepted: 10 February 2022 / Published online: 19 February 2022

Abstract

Non-alcoholic fatty liver disease (NAFLD) is rapidly being recognized as the leading cause of chronic liver disease worldwide. *Men1*, encoding protein of menin, is a key causative gene of multiple endocrine neoplasia type 1 syndrome including pancreatic tumor. It is known that insulin that secretes by endocrine tissue pancreatic islets plays a critical role in hepatic metabolism. Mouse model of hemizygous deletion of *Men1* was shown to have severe hepatic metabolism disorders. However, the molecular function of menin on lipid deposition in hepatocytes needs to be further studied. Transcriptome sequencing does show that expression suppression of *Men1* in mouse hepatocytes widely affect signaling pathways involved in hepatic metabolism, such as fatty acid metabolism, insulin response, glucose metabolism and inflammation. Further molecular studies indicates that menin overexpression inhibits expressions of the fat synthesis genes *Srebp-1c, Fas,* and *Acc1*, the fat differentiation genes *Pparγ1* and *Pparγ2*, and the fat transport gene *Cd36*, thereby inhibiting the fat accumulation in hepatocytes. The biological process of menin regulating hepatic lipid metabolism was accomplished by interacting with the transcription factor FoxO1, which is also found to be critical for lipid metabolism. Moreover, menin responds to insulin in hepatocytes and mediates its regulatory effect on hepatic metabolism. Our findings suggest that menin is a crucial mediation factor in regulating the hepatic fat deposition, suggesting it could be a potential important therapeutic target for NAFLD.

Keywords Menin · Hepatocyte · Lipid metabolism · FoxO1 · Non-alcoholic fatty liver disease (NAFLD)

Abbreviations

Acc1	Acetyl CoA carboxylase
Akt, also named as PKB	Protein kinase B
Cd36	A fatty acid transporter
FoxO1	Forkhead box O1
ChIP	Chromatin immunoprecipitation
DEGs	Differentially expressed genes
Fas	Fatty acid synthase
Gk	Glucokinase
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
MEN1	Multiple endocrine neoplasia type 1
NAFLD	Nonalcoholic fatty liver disease
OA	Oleic acid
Pparγ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SIRT1	Sirtuin 1
Srebp-1c	Sterol regulatory element-binding protein 1C
T2DM	Type 2 diabetes mellitus
TG	Triglyceride

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Introduction

Non-alcoholic fatty liver disease (NAFLD), defined as excess fat accumulation in the liver, is rapidly becoming the most common cause for chronic liver disease worldwide. Nevertheless, the current treatment of NAFLD, there is no appropriate drug treatment, mainly through diet and lifestyle intervention [1–3]. Therefore, there is an urgent need to identify key factors that regulate liver fat metabolism to develop therapeutic targets for NAFLD.

Menin is a 610 amino acid protein encoded by *Men1* gene on human 11q13 chromosome, which is a key causative agent of multiple endocrine neoplasia type 1 (MEN1) [4]. Menin has a tumor suppressing effect in the endocrine system [5–7]. Moreover, menin as a key scaffold protein interacts with a variety of transcription factors and participates in multiple biological processes, including metabolic processes [8–11]. Recent studies have shown that menin is involved in the glucose and lipid metabolism in liver. For example, liver-specific *Men1* hemizygous deletion ($Men1^{\pm}$) mutant mice show abnormal liver glucose tolerance and lipid metabolism after feeding on a high-fat diet [12]; Islet-specific *Men1* knockout mouse obtain increased pancreatic cell proliferation and insulin secretion [13–15]; Menin affects fatty acid uptake in liver cells through targeting *Cd36* gene transcription regulation[16]. Nonetheless, the mechanism by which menin regulates hepatic lipid metabolism is poorly understood.

It is well-known that the transcription factor forkhead box O1 (FoxO1) plays a crucial role in glucose and lipid metabolism [17]. Insulin enhances the interaction between menin and FoxO1 through Akt, therefore affecting the glucose metabolism in liver [18]. However, it is not clear whether menin binds to FoxO1 to regulate lipid metabolism. Therefore, the purpose of this study is to investigate the possible mechanism of menin regulates lipid metabolism, and whether it is interacting with FoxO1. Our study found that menin down-regulates lipid deposition in hepatocytes. This may be the consequent that menin interacts with FoxO1 targeting the transcription activities of *Cd36* and *Gk* promoter and thereby regulating lipid metabolism in liver.

Materials and methods

Cell culture

Immortalized mouse normal (healthy) hepatocytes (NCTC-1469) used in this study were purchased from the National Cell Resource Center (Beijing, China). Cells were grown in Dulbecco's modified Eagle's medium-F12 (DMEM/F12; Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Biological Industries, Israel), penicillin–streptomycin liquid (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in an atmosphere of 5% CO_2.

Cell transfection

A recombinant expression plasmid of *Men1* was constructed according to the *Mus musculus Men1* sequence (NM_001168489). The plasmid was designated as pcDNA3.1 (+)-mMen1, and pcDNA3.1(+)-vector was negative control plasmid. *Men1*- and *FoxO*1-specific short interfering (si) RNA (siG12315132207 and siB13415161059, designated as si-Men1 and si-FoxO1, respectively), as well as its negative control siRNA (siN05815122147, designated as NC), were designed and synthesized by Ribobio (Guangzhou, China). The cells were inoculated into 6-well plates and replaced with a fresh culture medium when there were approximately 1×10^6 cells in each well. Then, siRNA (50 nM) or plasmid (1.5 μg) was added and transferred by Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA and protein were isolated from the transfected cells 24 h post-transfection for further analysis.

Total RNA extraction and real-time PCR analysis

Total RNA was extracted using the RNASimple Total RNA Kit (Tiangen Biotech, Beijing, China) and reverse transcribed using PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Bio, Shiga, Japan). The extracted RNA was reverse transcribed according to the instructions of the kit. In the 20 μL reverse transcription system, the maximum amount of total RNA used was 1000 ng. Real-time quantitative PCR was performed on the ABI 7500 instrument using the SYBR®Premix Ex TaqTM (Perfect Real-Time) kit (TaKaRa Bio) using the SYBR Green I dye method. The primer sequences are listed in Supplementary Table S1. The expression levels of the target mRNAs were normalized to the internal control *β-actin* mRNA. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative abundance of mRNA. The results are representative of at least three independent experiments to determine the statistical significance.

Western blotting

After transfection of *Men1*-specific plasmid and/or siRNA, total protein was extracted from NCTC-1469 cells using RIPA lysis buffer (containing 1% phenylmethylsulfonyl fluoride) (Beyotime, Shanghai, China). The protein concentration was detected by the BCA kit (Beyotime). Protein (20 μg) were separated by 10% SDS-PAGE and transferred

to the PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated overnight with the primary antibodies menin (1:1000 dilution; Abcam, Cambridge, UK), FoxO1 (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA) or β-actin (1:1000 dilution; Beyotime). β-actin was used as a total protein loading control. It was incubated with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution; Beyotime) for 4 h at 4 °C. Chemiluminescence detection was performed using BeyoECL Plus (Beyotime). The data shows the level of expression normalized to the corresponding negative control. The results are representative of three independent experiments that were used to determine the statistical significance.

Gene expression analysis by RNA-Seq

Three sets of RNAs with lower expression level of *Men1* ($31\% \pm 4.6\%$, $n=3$) were sent for transcriptome sequencing (BaiMaiKe, Beijing, China). The RNA samples were quality guaranteed, followed by the construction of the library. The quality of the library was detected to meet the requirements before sequencing is done. The data were filtered to obtain Clean Data, and the data were compared with the specified reference genome. The resulting Mapped Data were used to evaluate the quality of the library, with attributes including the length of the inserted fragment, randomness of the fragment, discovery of new genes, and optimization of gene structure. Based on the expression level of all genes in different sample groups, the differences in the expression level of the genes were analyzed using Fold Change > 1.5 and false discovery rate (FDR) < 0.01 as the screening criteria. The functional differences of the differentially expressed genes (DEGs) were screened and the functional enrichment of different analytical methods was carried out.

Gene Ontology (GO) enrichment analysis

GO enrichment analysis of the DEGs was implemented by the GOseq R packages based Wallenius non-central hypergeometric distribution [13, 19, 20], which can adjust for gene length bias in DEGs. The GO annotations contains three parts: the Biological Process, Molecular Function, and Cellular Component. The DEGs were analyzed by GO enrichment, and the GO annotations of each DEGs could be obtained. The Web Gene Ontology (WEG) Annotation Plot was used for GO function classifications [21].

Kyoto Encyclopedia of Genes and genomes (KEGG) pathway enrichment analysis

KEGG is a database resource for understanding high-level functions and utilities of biological systems [22], such as cells, organisms, and ecosystems, from molecular level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. We used the KEGG Orthology Based Annotation System (KOBAS) software to test the statistical enrichment of differential expression genes in KEGG pathways [23].

Insulin treatment of NCTC-1469 cells

NCTC-1469 cells (8.0×10^5 cells/well) were inoculated in 6-well cell culture plates and incubated until the cells were 70% to 80% confluent. The blank control group was 10% FBS DMEM/F12 culture. Treated samples received insulin with concentration of 0.8, 2, 3.2, 4.8, 6 or 7.2 μg/mL. After continuous culture for 2, 4, 6, 8, 10, and/or 12 h, total intracellular RNA was extracted and RT-PCR was used to detect the changes in the expression level of *Men1* in mouse normal (healthy) hepatocytes. The concentration and time point that affected the most significant changes in the expression level of *Men1* mRNA was selected (Fig S1).

Oil Red O staining

The expression of menin was inhibited or enhanced by transfection of hepatocytes with siRNA (si-Men1) or plasmid (pcDNA3.1 (+)-mMen1). The cells in the 6-well cell culture plate was added to 1 mL of 4% paraformaldehyde per well and the cells were fixed for at least 30 min. The cells were stained with the Oil Red O Stain Kit (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions. After washing with 1 × PBS, the images were observed and photographed under a microscope.

Isopropanol was used to extract oil red O and spectrophotometry was tested for the relative quantitative analysis of intracellular lipids [24]. In brief, 200 μl of 100% isopropanol was added to each well to extract oil red O and the mixtures were incubated for 10 min at room temperature. Then, 100 μl was transferred to a 96-well plate. OD value (absorbance) at 490 nm wavelength was measured by a microplate reader.

Cellular triglyceride (TG) assay

The level of TG in the cells was detected by ELISA. The cells were collected and disrupted by ultrasonication. Part of the cell lysates were detected protein concentration by BCA (Beyotime), and the other cell lysates were used for determination of TG using the Triglyceride assay kit (Nanjing Jiancheng). TG content was calculated (mmol/gprot) as (sample optical density [OD] value − blank OD value) / (calibration OD value − blank OD value) × calibrator concentration (mmol/L) / sample protein concentration (gprot/L). Samples comprised the transfected control

group and the treatment group. The blank was 1 × PBS. The calibration product was provided as part of the kit.

Fatty hepatocyte model

Exposure of hepatocytes to oleic acid (OA) induces mRNA expression of enzymes involved in adipogenesis, accumulation of intracellular lipid droplets, and significantly increased triglyceride content, which has been widely used as a model of hepatocyte fatty liver [25, 26]. Therefore, hepatocytes were treated with oleic acid to establish the model of fatty hepatocytes. Cells were inoculated to a 96-well plate (1×10^4 cells/well). Cells were treated with different concentrations of OA (0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 mM/L), and cell viability was detected after 24, 48, or 72 h. 50 µL MTT was added to each well, and incubation was continued for 4 h. The supernatant was discarded, 150 µL of dimethylsulfoxide was added to each well. The absorbance value of each well was measured by a microplate reader at a wavelength of 570 nm. The survival rate was determined by the formula: cell viability (T/C% expressed) = (test group average OD value / control group average OD value) × 100%. After 24, 48, and 72 h of treatment with the optimal concentration of OA, TG content was detected and Oil Red O staining was done. Finally, the fatty hepatocyte model was validated (Fig S2).

Chromatin immunoprecipitation (ChIP)

NCTC-1469 cells ($1–2 \times 10^7$ cells) were cross-linked with 1% formaldehyde and lysed in 1 mL SDS lysis buffer. Then, the chromatin was sheared into 200–1000 bp fragments using a Bioruptor ultrasonic apparatus. For each immunoassay, ultrasound-treated cell lysates were filtered by agarosaccharide beads and 1% supernatant was taken as input. The remainder was divided into two tubes, and 1.6 µg anti-rabbit menin (Abcam) and anti-rabbit IgG antibodies (Cell Signaling Technology) were added for ChIP analysis. Precipitated DNA was used as a template for quantitative real-time PCR using sybr-premix Ex Taq II (TaKaRa Bio). The primer pair to screen the 1200 bp promoter region of *Gk* and *Cd36* was designed and synthesized. Details are provided in the supplemental data (Table S2). For quantitative real-time PCR, Ct values for each ChIP were obtained from triplicate reactions. The results represent three separate ChIP experiments, in which the amount of chromatin obtained from immunoprecipitation was quantified relative to the amount in the input sample, and the input percentage of chromatin obtained from immunoprecipitation was quantified relative to the amount in the input sample.

Statistical analysis

The results are expressed as mean ± SD. The difference was statistically analyzed using SAS 8.2 statistical software (SAS Institute, Cary, NC) for single factor analysis of variance (ANOVA). Significant difference was denoted $P < 0.05$ (*) and $P < 0.01$ (**).

Results

Inhibition of expression of menin in hepatocytes affects signaling pathways involved in glycolipid metabolism and inflammatory responses

To explore the role of menin in the liver, NCTC-1469 mouse normal hepatocytes were used as an in vitro hepatocyte model. Menin expression was knocked down by transfection of the cells with *Men1*-specific siRNA, After transfection for 24 h, the mRNA expression level of *Men1* in the si-Men1 group was 31% ± 4.6% ($n = 3$), compared to that in the negative control group (Fig. 1A). Triplicates of the menin expression inhibited cells, as well as their negative control cells, were submitted for transcriptome sequencing analysis. A total of 157 differentially expressed genes (DEGs) were identified, among which 51 genes were upregulated and 106 genes were down-regulated. Gene expression validation results of randomly selected genes (four down-regulated and eight upregulated genes) showed that the correlation coefficient (R^2) of qRT-PCR data with sequencing data was 0.916 (Fig. 1B), suggesting the accurate and reliable identification of DEGs upon lower expression of *Men1*.

KEGG enrichment analysis of DEGs identified pathways including the adipocytokine signaling pathway related to fat metabolism, tumor necrosis factor signaling pathway related to inflammation and glycolipid metabolism, and a variety of inflammatory pathways (Fig. 1C). GO analysis revealed the involvement of the DEGs in the insulin response, glucose metabolism, fatty acid metabolism, and inflammatory response (Table 1). These results indicated that modification of menin expression in mouse hepatocytes affects inflammation and glycolipid metabolism pathways, which are closely related to the occurrence of fatty liver disease in vivo [27].

Inhibition of menin expression promotes fat accumulation in hepatocytes

To further investigate whether menin has an effect on fat deposition in hepatocytes, *Men1* expression was inhibited by transfection of specific siRNAs (si-Men1) into mouse hepatocytes (NCTC-1469 cells, Fig. 2A, B). The inhibition of *Men1* caused significantly decreased expression of *Gk* gene, which was involved in glycolytic pathway (Fig. 2C).

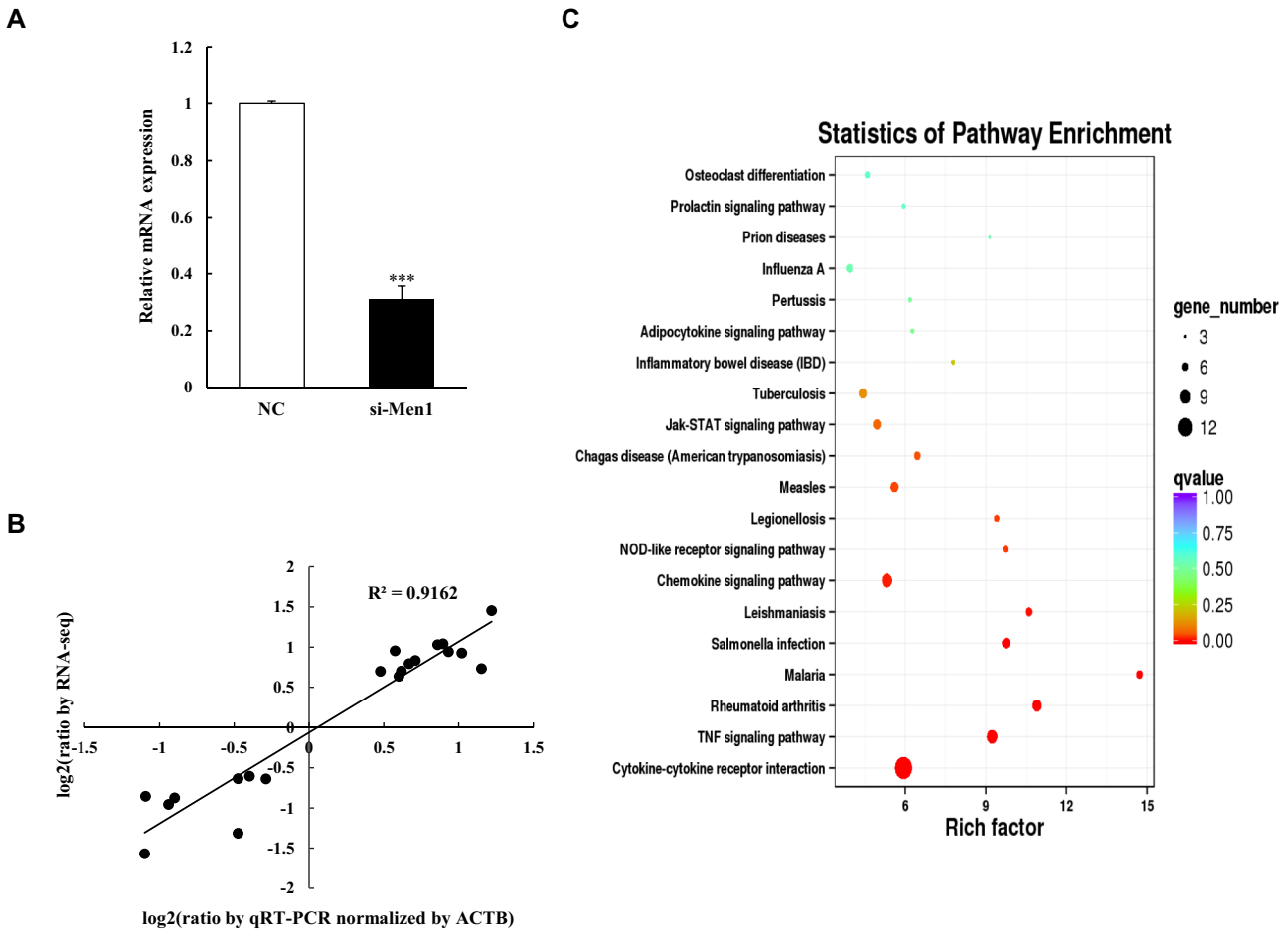

Fig. 1 Knockdown of *Men1* in hepatocytes causes changes in signaling pathways involved in glycolipid metabolism and inflammatory responses. **A** Mouse normal hepatocytes NCTC-1469 cells were transfected with Men1-specific siRNA (si-Men1) or control (NC) for 24 h, $n=3$. The expression level of *Men1* mRNA were reduced to 30.2% ± 0.58% via qRT-PCR. **B** Three sets of the *Men1*-knockdown RNA, as well as their control, were performed RNA-seq. The high correlation coefficient ($R^2=0.92$) of fold changes for representative differentially expressed genes between RNA-seq and qRT-PCR data indicated the present analysis was stable and repeatable. **C** KEGG analysis showed that the differentially expressed genes after *Men1*-knockdown enriched into signaling pathways such as glucose metabolism, lipid metabolism and inflammatory responses. Data are means ± SEM of 3 independent experiments per each group. ***$P<0.001$

However, the expression of the fatty acid synthesis genes *Srebp-1c*, *Acc1*, *Fas* and the fatty acid transporter gene *Cd36* were all significantly increased, as well as the expression of adipose differentiation related gene *Pparγ1* and *Pparγ2* [28, 29].

To further validate the effect of menin on lipid metabolism in mouse hepatocytes, we examined the triglyceride (TG) content after knockdown of menin. The TG content was significantly increased upon menin expression inhibition compared to the negative control (Fig. 2D). Moreover, to detect whether the content of lipid droplets changed, we stained the transfected cells with Oil Red O and extracted with absolute ethanol to determine the absorbance value. The menin knockdown cells showed significant increase absorbance value, indicating significant increase in lipid droplet content (Fig. 2E). These results suggested that the decreased expression of menin enhances fat synthesis, transport and deposition in mouse hepatocytes.

Overexpression of *Men1* inhibits fat accumulation in hepatocytes

To further verify the effect of menin on lipid metabolism, hepatocytes were transfected with the *Men1*-overexpressed plasmid pcDNA3.1(+)-m*Men1* to examine its metabolic effects. After transfection, *Men1* mRNA and menin protein levels were significantly elevated compared to vector controls (Fig. 3A, B). Enhanced expression of menin caused significantly increased expression of *Gk*. Moreover, the expression of *Fas* and *Srebp-1c* were decreased ($p=0.081$

Table 1 GO enrichment of differentially expressed genes upon *Men1* expression inhibition, indicating critical regulatory role of *Men1* in hepatocyte metabolism

Category	GO:ID	Biological process	Involved genes	KS[a]
Insulin response	GO:0,032,869	Cellular response to insulin stimulus	*Egr1, Sgk1*	1.10E-04
	GO:0,046,676	Negative regulation of insulin secretion	*Prkaa2*	7.50E-04
	GO:0,050,796	Regulation of insulin secretion	*Il1b*	2.66E-03
	GO:0,032,868	Response to insulin	*Tlr2*	8.96E-03
	GO:0,030,073	Insulin secretion	*Anxa2*	2.60E-02
Glucose metabolism	GO:0,071,333	Cellular response to glucose stimulus	*Il1b*	1.12E-03
	GO:0,009,749	Response to glucose	*Thbs1, Egr1*	4.70E-03
	GO:0,042,593	Glucose homeostasis	*Prkaa2, Serpine1*	5.67E-03
	GO:0,035,774	Positive regulation of insulin secretion involved in cellular response to glucose stimulus	*Arrb1*	1.57E-02
	GO:0,042,149	Cellular response to glucose starvation	*Prkaa2*	3.64E-02
	GO:0,010,829	Negative regulation of glucose transport	*Il1b*	5.92E-02
Fatty acid metabolism	GO:0,006,635	Fatty acid beta-oxidation	*Acsbg1*	3.50E-06
	GO:0,006,633	Fatty acid biosynthetic process	*Prkaa2*	9.30E-04
	GO:0,010,746	Negative regulation of plasma membrane long-chain fatty acid transport	*Thbs1*	5.37E-03
	GO:0,000,038	Very long-chain fatty acid metabolic process	*Cyp4f18, Acsbg1*	1.23E-02
	GO:0,070,542	Response to fatty acid	*Ptgs2, Tlr2*	1.96E-02
	GO:0,071,398	Cellular response to fatty acid	*Il1b*	3.12E-02
Inflammation	GO:0,002,439	Chronic inflammatory response to antigenic stimulus	*Orm1, Orm2, Il1b*	1.75E-03
	GO:0,050,728	Negative regulation of inflammatory response	*Irg1, Ctla2a, Tyro3, Ier3*	1.88E-03
	GO:0,050,727	Regulation of inflammatory response	*Slc7a2*	3.54E-03
	GO:0,006,954	Inflammatory response	*Il18, Ccl7, Fpr1, Ccl2, Aif1*	1.13E-02
	GO:0,050,729	Positive regulation of inflammatory response	*Tgm2, Tlr2, Jak2, Ccl24, Osbpl3*	3.67E-02
	GO:0,002,677	Negative regulation of chronic inflammatory response	*Tnfaip3*	5.53E-02
	GO:0,002,526	Acute inflammatory response	*Ptges, Cxcl3, Cxcl2,*	8.02E-02
Other	GO:0,007,568	Aging	*Fos, Il1b, Fosl2, Cp, Socs3, Ccl2, Ccl24, Ass1*	2.60E-10
	GO:0,055,114	Oxidation–reduction process	*Ptgs2, Cxcl2, Cyp4f18, Plod2, Mica12, Cp, Cxcl3, Kdm5b*	1.60E-07
	GO:0,001,889	Liver development	*Hp, Ass1, Cp, Ephx1*	2.01E-04

[a]*KS* statistically enrichment significance of the GO term. Smaller KS value indicates more significant enrichment of the GO term

and $p = 0.052$, respectively), and the expression of *Acc1, Cd36, Pparγ1* and *Pparγ2* were obviously decreased ($p < 0.05$) (Fig. 3C). These results were opposite to those observed with that of menin inhibition, suggesting that an increased expression of menin has an inhibitory effect on fat synthesis. This was further verified by two different assays. Firstly, the amount of TG was observably reduced upon menin overexpression compared to negative controls (Fig. 3D). Furthermore, to detect whether menin could rescue the fat deposition in fatty hepatocytes induced by Oleic acid (OA), Oil Red O staining was used to assess the content of lipid droplets. The measurement of the absorbance due to the stain showed that the overexpression of menin reduced the fat deposition in OA-induced hepatocytes ($p > 0.05$), indicating that menin suppresses the fat deposition (Fig. 3E).

Menin mediates the effect of insulin on lipid metabolism by responding to insulin surrounding the hepatocytes

Insulin is important in regulating cell metabolism of different tissues, including the liver. To test the effect of insulin on *Men1* expression in hepatocytes, NCTC-1469 cells were treated with insulin. The results showed that *Men1* expression was gradually suppressed dependent on the insulin concentration (Fig S1), with the lowest *Men1* expression level observed at 6 μg/mL insulin at 8 h post treatment (Fig. 4A). The expression levels of *Pparγ1, Pparγ2, Cd36*, *Srebp-1c*, *Fas* and *Acc1* were significantly elevated (Fig. 4B). These results suggest that menin may be involved in the regulation of insulin on hepatocyte metabolism.

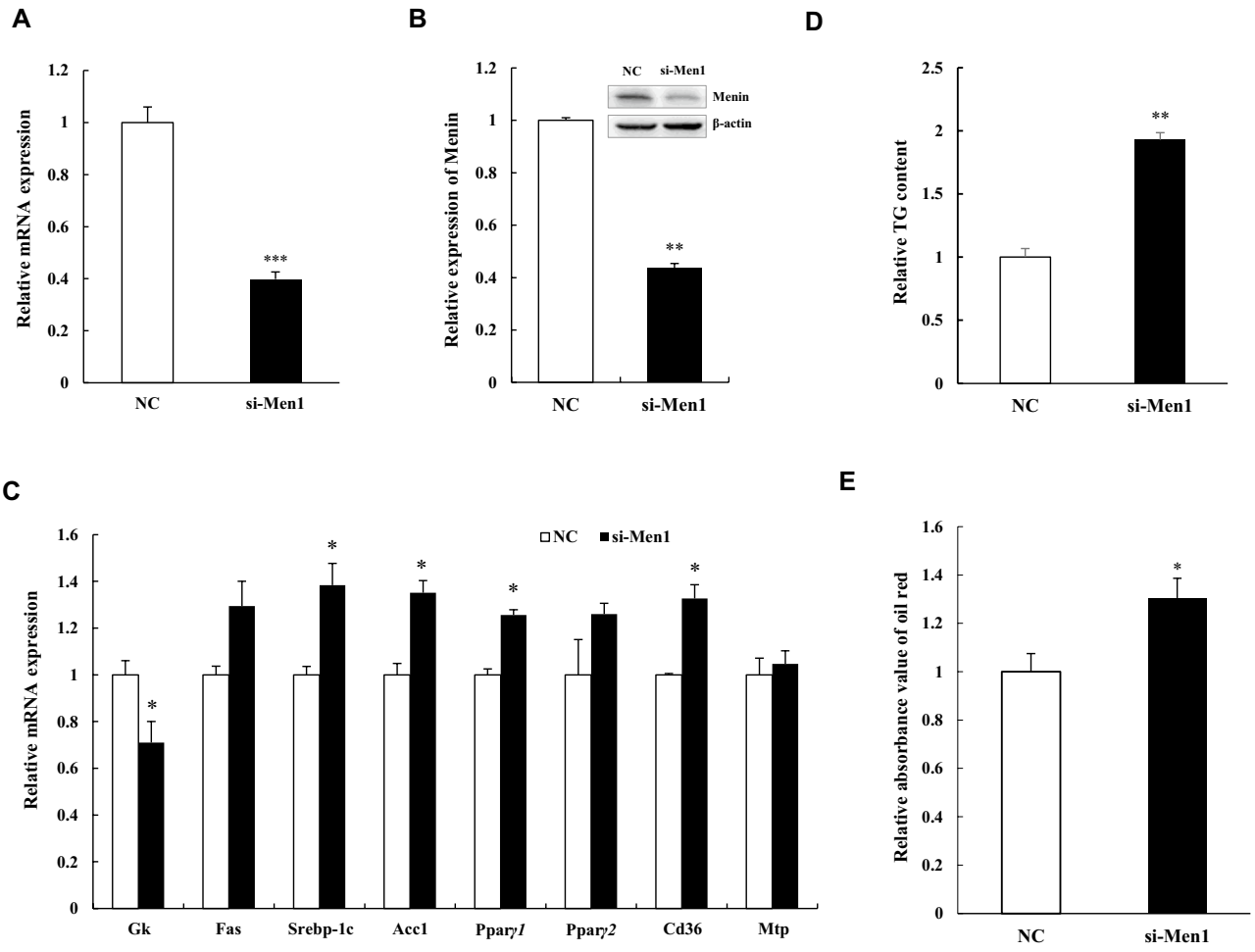

Fig. 2 The knockdown of *Men1* promotes fat accumulation in hepatocytes. NCTC-1469 cells were transfected with Men1-specific siRNA (si-Men1) or control (NC) for 24 h. **A**, **B** The expression levels of *Men1* mRNA (**A**) and menin protein (**B**) were detected via qRT-PCR and western blotting. **C** After knockdown of *Men1*, the expression of genes related to lipid synthesis, differentiation and fat transport was changed via qRT-PCR. **D** After knockdown of *Men1*, the cellular triglyceride (TG) were significantly increased by ELISA. **E** The lipid drops were increased in *Men1*-knockdown hepatocytes via absorbance value of the extracted oil red O dye at 490 nm. Data are means ± SEM of 3 independent experiments per each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Menin associates with the promoters of *Cd36* and *Gk* gene in hepatocytes

Based on the above results, we found no matter menin expression was enhanced or inhibited, the expression of *Cd36* and *Gk* would be affected (Figs. 2C and 3C). Thus, we hypothesized that menin might recruit transcription factors and associate with promoter regions of target genes, modulating their transcriptions. Thus, a ChIP assay was performed in hepatocytes. We initially designed four amplicons (P1, P2, P3, and P4) to determine whether menin binds to the promoter regions of *Cd36* and *Gk*. (Fig. 5A, C). It was found that menin bound to the regions detectable by P1 ($P = 0.18$), P2 ($P = 0.19$), P3 ($P = 0.18$) and P4 ($P = 0.014$) for *Cd36* gene (Fig. 5B). Whereas, for *Gk* gene, menin bound to the regions detectable by P1 ($P = 0.28$), P2 ($P = 0.22$), P3 ($P < 0.01$) and P4 ($P < 0.01$). These results suggested that menin may regulate lipid metabolism and glycolysis in hepatocytes by binding to the *Cd36* and *Gk* promoters.

Menin and FoxO1 interact and co-regulate lipid metabolism in hepatocytes

FoxO1 has been shown to play a crucial role in glucose and lipid metabolism in the liver [17]. To investigate the effect of *FoxO1* on metabolism-related genes in hepatocytes, the expression of *FoxO1* was inhibited by transfection of si-Foxo1 and followed by detection of target gene expression. The results showed that inhibition of *FoxO1* expression (39.3% ± 3.6% of control cells, $n = 3$) decreased the expression of *Srebp-1c, Acc1, Fas*, *Cd36, Pparγ1*, *Pparγ2* and *Mtp*, but increased the expression of *Gk* (Fig. 6A). Moreover, the

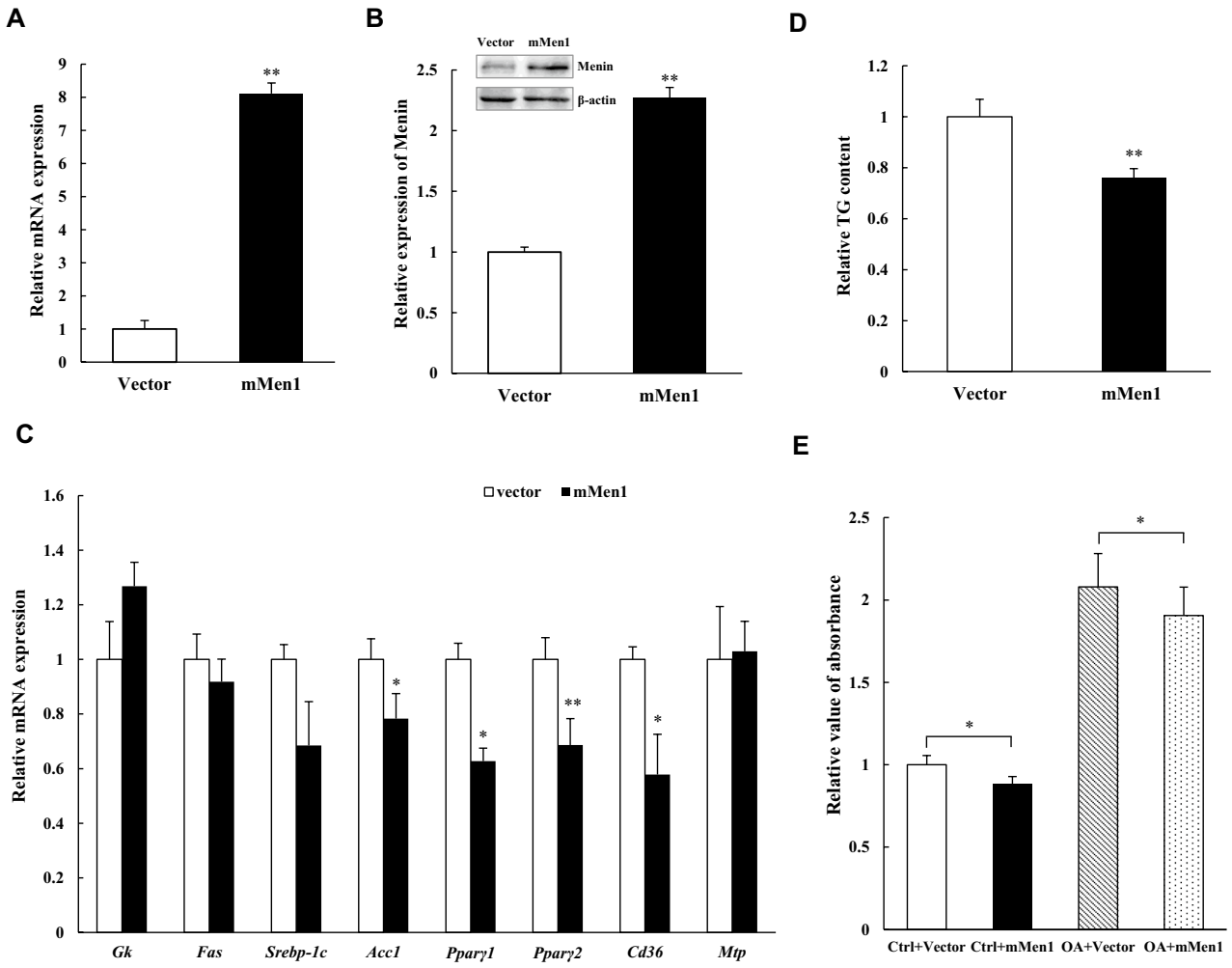

Fig. 3 The overexpression of *Men1* inhibits fat accumulation in hepatocytes. NCTC-1469 cells were transfected with pcDNA3.1(+)-mMen1 (mMen1) or control (Vector) for 24 h to overexpress Men1. **A**, **B** The expression levels of *Men1* mRNA (**A**) and menin protein (**B**) were detected via qRT-PCR and western blotting. **C** After overexpression of *Men1*, the expression of genes related to lipid synthesis, differentiation and fat transport was changed via qRT-PCR. **D** After overexpression of *Men1*, the cellular triglyceride (TG) were significantly decreased by ELISA. **E** NCTC-1469 cells were divided into 4 groups: normal (healthy) cells were transfected with Vector (Ctrl + Vector); normal cells were transfected with mMen1 (Ctrl + mMen1); Fatty liver model cells were transfected with Vector (OA + Vector); Fatty liver model cells were transfected with mMen1 (OA + mMen1). The cellular lipid drops were decreased after overexpression of *Men1* via absorbance value of the extracted Oil Red O dye at 490 nm. Data are means ± SEM of 3 independent experiments per each group. $^{*}P < 0.05$, $^{**}P < 0.01$

knockdown of FoxO1 significantly reduced lipid deposition in both healthy and fatty liver cells (Fig. 6B). Thus, Both menin and FoxO1 can regulate the same target gene expression, but in an opposite regulatory direction (Figs. 2 and 3), indicating a coordinating acting model of menin and FoxO1 in controlling the lipid metabolism in mouse hepatocytes. This model was confirmed by co-IP assays in both menin overexpressed and knockdown NCTC-1469 cells. The results showed that the amount of FoxO1 binding to menin was significantly increased upon menin knockdown, while, significantly decreased upon menin overexpression (Fig. 6C, D).

Discussion

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, accounting for approximately 20% ~ 30% of the global prevalence [30]. NAFLD is associated with a variety of metabolic disorder, mainly fat deposition in liver, but the critical molecular determinants of its pathogenesis remain largely unexplored. Although menin is a focus of tumor-associated research, it is seldom studied in lipid and glucose metabolism in liver. Our collective results indicate that menin plays an important role in regulating glucose metabolism and lipid metabolism in the liver. Altered

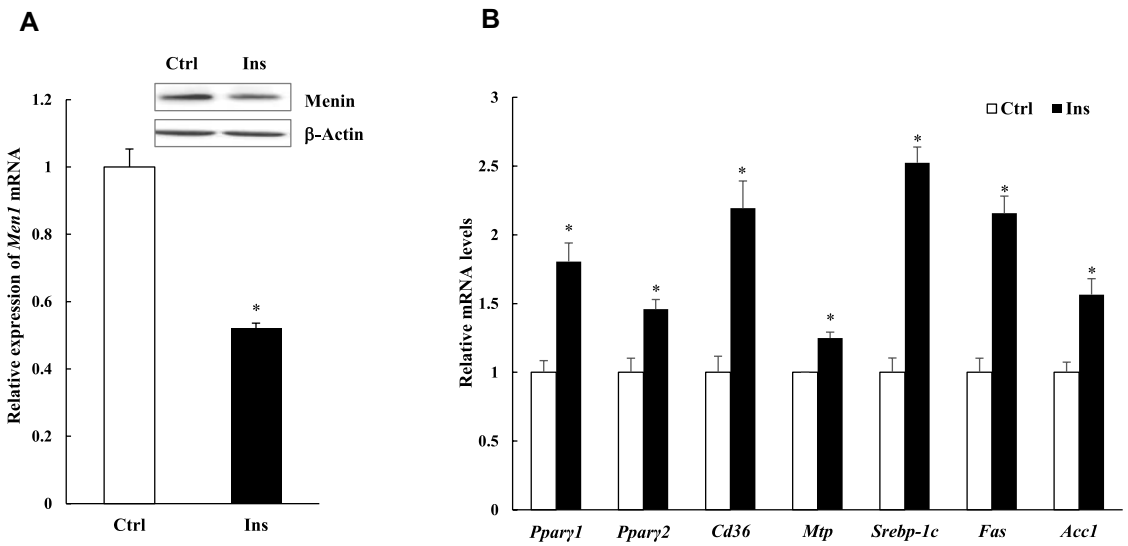

Fig. 4 Insulin-treated hepatocytes inhibits *Men1*/menin expression and promotes fat accumulation. NCTC-1469 cells were treated with 6 μg/mL insulin or negative control PBS (Ctrl) for 8 h. **A** The expression level of *Men1* mRNA and it protein menin were both detected to be inhibited compared with control, via qRT-PCR and western blotting. **B** After insulin treatment, the expression of genes related to lipid synthesis, differentiation and fat transport was up-regulated. Data are means ± SEM of 3 independent experiments per each group. $^*p < 0.05$

menin expression in mouse hepatocytes can cause abnormalities including glucose metabolism, lipid metabolism and inflammatory response (Table 1, Fig. 1). Furthermore, overespression of menin inhibits triglyceride deposition in hepatocytes by regulating the expression of genes involved in fatty acid differentiation, synthesis and transport in hepatocytes (Figs. 2 and 3), indicating menin's important role in maintaining normal liver function. The function of menin in lipid metabolism is consistent with previous reports, and fat deposition were increased in hepatocytes after menin knockdown [12, 16].

Menin regulates the lipid metabolism by coordinating with FoxO1 and targeting the same gene expression that are closely associated with fat deposition

We found that overexpression of menin suppressed the expression of genes related to fat differentiation (*Pparγ1* and *Pparγ2*), transport (*Cd36*) and synthesis (*Srebp-1c*, *Acc1*, and *Fas*) in hepatocytes, while increased the expression of the key enzyme gene for glycogen synthesis genes (*Gk*) (Fig. 3). Meanwhile, menin overexpression reduced the TG content in hepatocytes, and attenuated the fat deposition in fatty hepatocytes (Fig. 3). And, the inhibition of menin had the opposite effect in hepatocytes (Fig. 2). Furthermore, we found that menin can bind to promoter regions of *Cd36* and *Gk* gene in hepatocyte (Fig. 5). Many studies have shown that inhibiting the expression of genes related to glucose production, including *Gk*, is essential for adipogenesis [31]. Interestingly, previous study also found that menin can deacetylate histone H3 by binding to the promoter region of *Cd36* via recruiting NAD-dependent deacetylase sirtuin 1 (SIRT1), thereby inhibiting the expression of *Cd36* and reducing the uptake of fatty acids by hepatocytes [16]. These results demonstrate that menin can modulate the lipid metabolism in hepatocytes by binding to the promoter region of lipid metabolism-related genes, such as *Cd36*, *Gk*.

Menin usually plays its regulatory role through acting as a key scaffold protein that interacts with a variety of proteins, such as transcription factors [8–11]. It is well-known that the transcription factor FoxO1 is involved in lipid metabolism and plays a critical role in the development of NAFLD [17, 32]. Moreover, overexpression of *FoxO1* in mice promotes liver fat deposition [33, 34]. Intriguingly, our results found inhibition of FoxO1 significantly reduced the fat deposition in both healthy and fatty hepatocytes (Fig. 6B), which was exactly consistent with the effect of menin overexpression (Fig. 2D, E) by targeting the same gene expression (Figs. 2C, 3C and 6A). Studies have shown that FoxO1 binds to the *Gk* and/or *Cd36* promoter region and regulates its expression [35, 36]. Here we also found that menin binds to the *Gk* and/or *Cd36* promoter region thereby regulating their expression, through interacting with FoxO1 in mouse hepatocytes (Fig. 6B). Thus, it suggests that menin regulates the lipid metabolism in hepatocytes by interacting with transcription factor FoxO1 and targeting the expression of lipid metabolism-related genes, including *Cd36*, *Gk*.

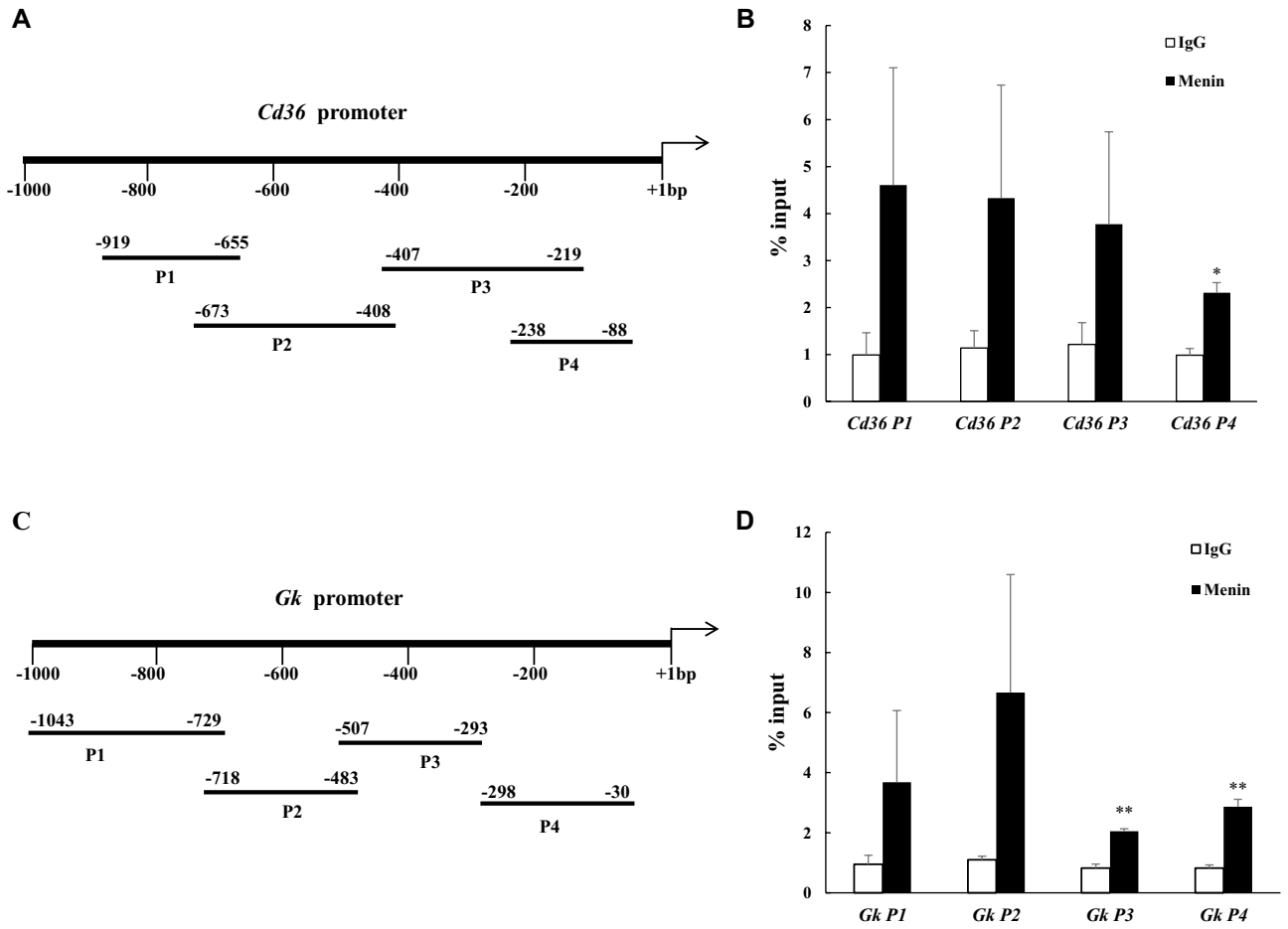

Fig. 5 *Cd36* and *Gk* are transcriptional targets of menin. **A** and **B** Four amplicons (P1, P2, P3, and P4) were designed to detect the indicated regions (upstream 1000 bp) of the *Cd36* (**A**) or *Gk* (**C**) by menin-ChIP-PCR assays in NCTC-1469 cells. **B** and **D** ChIP-qPCR analysis of menin binding to *Cd36* (**B**) or *Gk* (**D**) promoter. The results showed as the percentage of input by quantifying the amount of chromatin obtained from immunoprecipitation relative to the amount in the input samples. Data are means ± SEM of 3 independent experiments per each group. $^{**}P < 0.01$, $^{*}P < 0.05$

Menin mediates the hepatic lipid deposition by responding to insulin in hepatocytes

Results in this study also showed that insulin can significantly reduced the *Men1* expression and up-regulated the expression of lipid metabolism-associated genes (Fig. 4) that were also targeted by menin (Fig. 2C). The mechanism of insulin targeting the expression of menin in hepatocytes was also found present in mammary epithelial cells [37]. Furthermore, it was also demonstrated that insulin could regulated the glucose metabolism of liver through FoxO1 [38]. Meanwhile, Wuescher et al. found that insulin regulated the localization of menin in the cytoplasm and the interaction ability with FoxO1 in hepatocytes by Akt [18]. Interestingly, menin can suppress the Akt activity and regulate its cellular localization [38]. In addition, it was found the expression of FoxO1 was controlled by menin in hepatocytes, with promoted transcription upon menin inhibition and inhibited transcription upon menin overexpression (Fig S3). We presumed that there was a negative feedback loop in hepatocytes between menin and FoxO1, which had been found present between menin and miR-24-3p in mammary epithelial cells, too [11]. For example, overexpression of menin inhibited the transcription activity of FoxO1, causing attenuated binding amount of FoxO1 recruited by the adaptor protein menin, and thus inhibited fat deposition. Taken together, these results suggest that menin acts an important mediator in hepatocytes that responds to insulin, modulating its recruitment ability of binding to FoxO1, therefore regulating the activity of genes related to lipid metabolism, such as *Cd36* and *Gk*, and ultimately affects fat deposition in the liver (Fig. 7). A rescue experiment was performed and confirmed this model (Fig S4). The increased expression of *Cd36* in insulin treated cells tends to be balanced to normal level (negative control) upon menin over-expression (Ins + mMen1). However, it is worthy to mention that

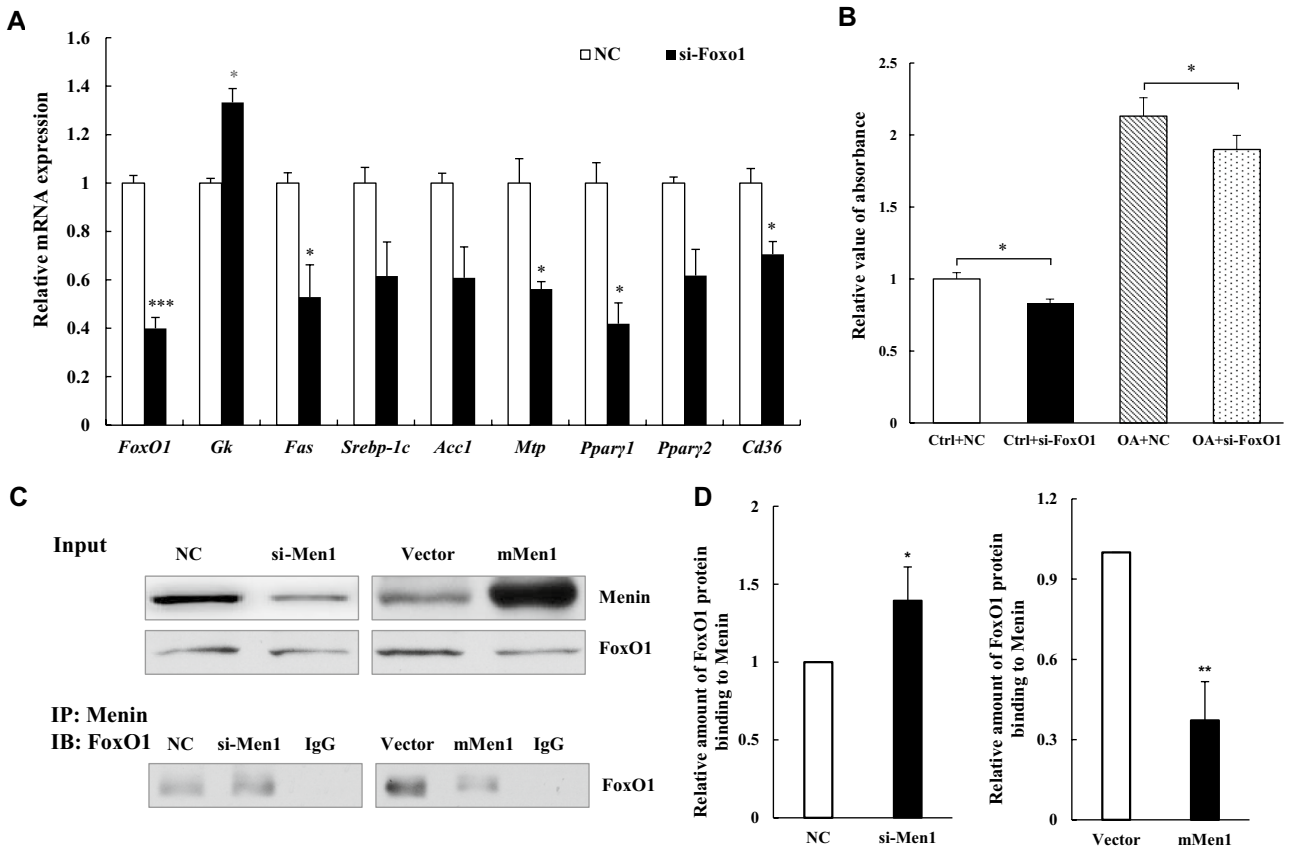

Fig. 6 Menin regulates the expression of genes involved in lipid metabolism by interacting with FoxO1. **A** NCTC-1469 cells were transfected with si-FoxO1 or negative control (NC) for 24 h to suppress FoxO1. Then, the expression of genes related to lipid synthesis, differentiation and fat transport was changed via qRT-PCR. **B** NCTC-1469 cells were divided into 4 group: normal (healthy) cells were transfected with NC (Ctrl + NC); normal cells were transfected with si-Foxo1 (Ctrl + si-Foxo1); Fatty liver model cells were transfected with NC (OA + NC); Fatty liver model cells were transfected with si-Foxo1 (OA + si-Foxo1). The cellular lipid drops were decreased after overexpression of *Men1* via absorbance value of the extracted Oil Red O dye at 490 nm. **C** Menin-immunoprecipitation (IP) assay were conducted in *Men1*-knockdown (si-Men1) and *Men1*-overexpressed (mMen1) NCTC-1469 cells, as well as their negative control cells (NC and Vector), together with IgG-IP control experiments. Foxo1-immunoblot assay results indicated that Menin could bind to FoxO1, not only in *Men1*-knockdown (si-Men1) and/or *Men1*-overexpressed (mMen1) cells but also in their negative control cells. (D) The quantitative results showed that the combination of menin and Foxo1 was significantly increased upon *Men1* expression inhibition (si-Men1) compared with negative control (NC). However, the combination of menin and Foxo1 was significantly decreased upon *Men1*-overexpressed (mMen1). Data are means ± SEM of 3 independent experiments per each group. $^{**}P < 0.01$, $^{*}P < 0.05$

menin might not be the only mediator that responds to insulin in hepatocytes. In another word, insulin might control the glucolipid metabolism in hepatocytes through different ways, since we observed that the expression levels of genes changed more dramatically upon insulin treatment than that happened upon menin inhibition (Figs. 4B and 2C).

Although our research revealed part of the mechanism by which menin regulates lipid metabolism in hepatocytes, this study has only been explored in vitro and needs to be studied in mouse models in the future. Although the commonly used NASH mouse model (diet-induced, genetic or a combination of more than one intervention) causes lipid accumulation in the liver and histological appearance is similar to that of NASH patients, the proteomics, lipidomic and transcription Omics is not similar to NASH patients [39, 40]. Studies have shown that the inbred isogenic offspring of a C57BL/6J mouse crossed with a s129/SvlmJ mouse showed obesity, insulin resistance, hypoadiponectinemia, adipose tissue inflammation, dyslipidemia, cell signaling, transcriptomic and lipidomic NASH patients [39, 41]. This NASH mouse model may be widely used in the future.

In summary, our study is the first to show that menin inhibits lipid deposition in hepatocytes by binding to *Cd36* and *Gk* promoter regions associated with lipid metabolism. Moreover, menin interacts with the transcription factor FoxO1 and may participate in insulin-mediated hepatic lipid metabolism by regulating the expression of target genes related to lipid metabolism. Our findings provide

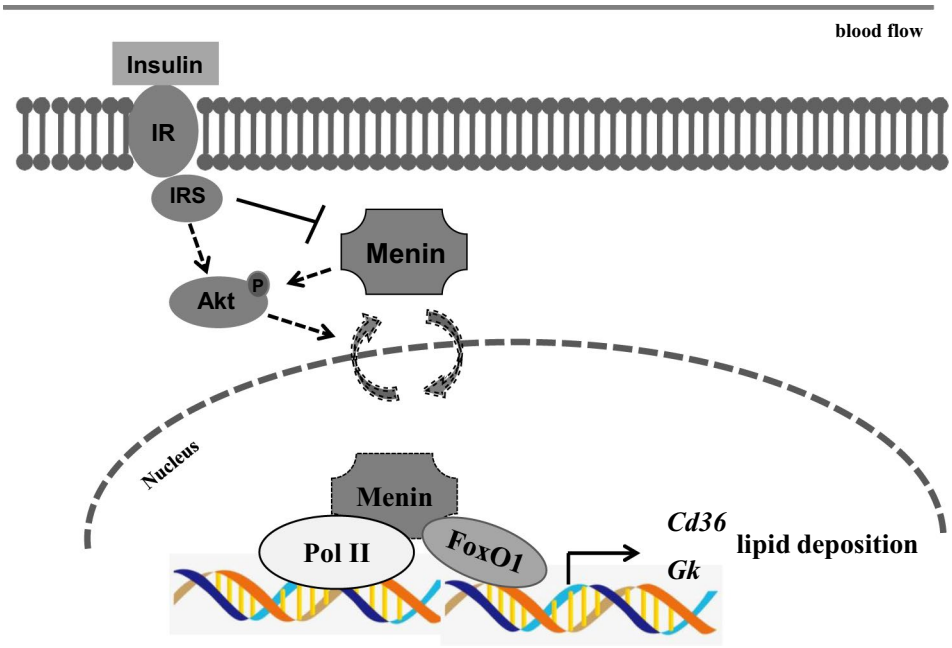

Fig. 7 Menin mediates hepatic metabolism through interacting with transcription factor FoxO1. Menin acts an important mediator in hepatocytes that responds to insulin, modulating its recruitment ability of binding to FoxO1, therefore regulating the activity of genes related to lipid metabolism, such as *Cd36* and *Gk*, and ultimately affects fat deposition in the liver. Please see the text for further details. *IR* insulin receptor, *IRS* IR substrate, *Akt* also known as protein kinase B, *Pol II* RNA polymerase II, *Cd36* a fatty acid transporter, *Gk* Glucokinase. The solid line represents fluxes; The dotted line represents possible effects or fluxes

new insights into the mechanism of menin liver lipid metabolism and may provide a theoretical basis for the clinical treatment of NAFLD patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11010-022-04392-6.

Acknowledgements We are grateful to Prof. Shaorong Gao in Tongji University for his helpful discussions and excellent suggestions for the project and manuscript. We also thank all present and past members of our laboratory who have contributed comments and ideas.

Author contributions Shi KR, Wang SX; Liu TJ and Du HX participated in the execution of all experiments, analyzed the data, and drafted the manuscript; Liu TJ, Sun LL, Xu ZJ, and Li RR participated in construction of the expression plasmids, data analysis and discussion; Yu Y and Mao YJ participated in experimental design, coordinated the experiments. All authors reviewed and approved the final manuscript.

Funding This work was financially supported by the Natural Science Foundation of Shandong (ZR2020MC166; ZR2013CM013), the National Key Research and Development Program of China (2021YFD1200900), the National Natural Science Foundation of China (31402054), the Key Project of Agricultural Fine Breeding of Shandong Province (2019LZGC011, 2016LZGC030).

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

Declarations

Conflict of interest The authors declare that they have no conflicts of interest concerning this article.

Ethical approval All process was completed on the basis of the Guidelines for Care and Use of Laboratory Animals of Shandong Agricultural University and approved by the Animal Ethics Committee of Shandong Agricultural University.

Consent for publication Not applicable.

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