Nonalcoholic fatty liver disease (NAFLD) is due to the excessive lipid accumulation within hepatocytes. Metabolic nuclear receptors (MNRs) play great roles in lipid homeostasis. We have identified a novel long noncoding RNA (lncRNA), lnc-HC, which regulates hepatocytic cholesterol metabolism through reducing Cyp7a1 and Abca1 expression. Here, we further elucidate its roles in hepatic fatty acid and triglyceride (TG) metabolism through a novel lncRNA regulatory mechanism. The most prominent target of lnc-HC identified by in vitro study is PPARγ. Further studies revealed that lnc-HC negatively regulates PPARγ at both the mRNA and protein levels and suppresses hepatocytic lipodroplet formation. Importantly, the function of lnc-HC in regulating PPARγ expression depends on modulating miR-130b-3p expression from the transcriptional to the post-transcriptional level, not through lncRNA’s critical modulating patterns. In vivo, the reduction of lnc-HC expression significantly decreases miR-130b-3p expression, induces PPARγ expression, and increases TG concentration in rat livers with hyperlipidemia. These findings further help in understanding the regulatory pattern of lnc-HC in hepatic lipid metabolism and might present a possible therapeutic target for improving lipid homeostasis.

INTRODUCTION
Nonalcoholic fatty liver disease (NAFLD) is one type of fatty liver disease that occurs in liver as lipid accumulation and hepatic steatosis due to causes other than alcohol uptake. NAFLD ranges from nonalcoholic fatty liver to nonalcoholic steatohepatitis, hepatic fibrosis, and cirrhosis, and can even become hepatic carcinoma. Furthermore, NAFLD is also closely associated with type II diabetes, atherosclerosis, and cardiovascular disease. Recent studies indicate that over 30% of people in the United States and up to 25% of adults worldwide have NAFLD. Although a growing number of studies help us understand the pathogenesis of NAFLD, we still lack an approved therapeutic regime due to its complexity. Thus, exploring the pathological process of NAFLD and discovering the critical drug targets could greatly contribute to understanding the molecular mechanism and supplying promising therapeutic candidates.

NAFLD manifests hepatocellular lipid deposits and injury as the main pathological change, which is caused by lipid disorder. Metabolic nuclear receptors (MNRs), as a big class of transcription factors (TFs), are critical contributors to energy and lipid homeostasis. MNR family contains peroxisome-proliferator-activated receptors (PPARs), liver X-activated receptors (LXRs), and the farnesoid X receptor (FXR). PPARs mainly participate in lipogenesis and insulin resistance. PPARγ powerfully regulates fatty acid uptake and storage and has been implicated in the pathology of obesity, diabetes, and atherosclerosis. Although PPARγ is a well-known therapeutic target in the treatment of hyperlipidemia and hyperglycemia in type 2 diabetes, PPARγ agonist causes adverse events such as liver function disorder and weight gain.

Existing studies have identified important roles for long noncoding RNAs (lncRNAs) in lipid metabolism. Ectopic expression of the lncRNA HOTAIR in abdominal preadipocytes could upregulate adipogenic gene expression of PPARγ and lipoprotein lipase (LPL) and induce the adipocyte differentiation. Bnc1 interacts with transcription factor EBF2 and promotes brown and beige adipocyte differentiation, which is linked to thermogenesis and systemic energy homeostasis. The lncRNA InclSTR regulates lipid homeostasis through TDP-43/FXR/apoC2 molecular pathways.
In our previous study, we identified a novel lncRNA, Inc-HC (a lncRNA derived from hepatocytes), differentially expressed in NAFLD rat livers. Inc-HC directly binds the co-regulator hnrNPA2B1 and further interacts with cholesterol catabolic gene Cyp7a1 or Abca1 as a lncRNA-protein-mRNA complex. Inc-HC regulates Cyp7a1 and Abca1 expression through hnrNPA2B1 and, thus, modulates hepatic cholesterol catabolism. Based on our previous work, we further identified the function of Inc-HC in controlling hepatic fatty acid and triglyceride (TG) metabolism. Our present work suggests that Inc-HC negatively regulates PPARγ expression at the post-transcriptional level through the mediator miR-130b-3p; the Inc-HC/miR-130b-3p/PPARγ pathway delicately regulates the accumulation of hepatic lipid droplet.

RESULTS

Inc-HC Participates in Hepatocytic Fatty Acid Metabolism

We first induced the lipid accumulation cell model by treating CBRH-7919 and BRL3A cells with mixed free fatty acids (FFAs; 1 mM; palmitic acid [PA], oleic acid [OA]; PA:OA, 1:2) for 24 h. Oil red O staining showed obvious formation of lipid droplets in CBRH-7919 cells (Figure 1A). Meanwhile, quantitative real-time PCR results showed the expression change of the lipid metabolic enzymes fatty acid synthase (Fasn; 80% decrease), carnitine palmitoyl transferase 1A (Cpt-1a; over 7-fold increase), and diacylglycerol O-acyltransferase 2 (Dgat2; 1.65-fold increase) and the expression change of the lipid MNRs PPARγ (25% decrease), PPARα (1.8-fold increase), FXR (2.1-fold increase), and Srebp1c (70% decrease) in the lipid accumulation cell model (Figures 1B and 1C). We observed a time-course-dependent increase of lnc-HC expression change of the lipid metabolic enzymes fatty acid synthase (Fasn; 25% decrease), PPARγ (1.65-fold increase) and the expression change of the lipid MNRs PPARγ (25% decrease), PPARα (1.8-fold increase), FXR (2.1-fold increase), and Srebp1c (70% decrease) in the lipid accumulation cell model (Figures 1B and 1C). We observed a time-course-dependent increase of lnc-HC and stable-knockdown cell lines, respectively named overLnc-HCCBRH and Lnc-HCshRCBRH. In overLnc-HCCBRH cells, mRNA expression of PPARγ and LXRxα was significantly decreased (Figure 2A). In Lnc-HCshRCBRH cells, only PPARγ mRNA was increased (Figure 2B). Western blotting analysis showed consistent results that Inc-HC could negatively regulate PPARγ protein expression (Figure 2C). Also, Inc-HC negatively regulated PPARγ expression both at the mRNA and the protein levels in BRL3A cells (Figures S2A and S2B). PPARγ comprehensively controls the TG synthesis and storage process. Here, quantitative real-time PCR analysis showed that TG-synthesis-associated genes, including Acc2, AcsL1, and Dgat2, were negatively regulated by Inc-HC and that their expressions were consistent with PPARγ variation (Figure 2D). In addition, Inc-HC negatively regulated FFA uptake genes Fatp-1 and aP2 and the FFA β-oxidation gene Cpt-1a (Figure 2D). With the transfection of PPARγ small interfering RNA (siPPARγ; GenePharma, Shanghai, China) in Lnc-HCshRCBRH cells, PPARγ expression was knocked down at the mRNA and protein levels (Figures 2E and 2F); meanwhile, the expression of PPARγ downstream genes, including aP2, Acc2, Dgat2, and Fatp-1, which were originally increased with Inc-HC knockdown, was decreased as compared with the negative control (NC) small interfering RNA group (siNC; GenePharma, Shanghai, China) (Figure 2G). Here, we confirmed that Inc-HC negatively regulated the expression of PPARγ and its pathway genes.

Inc-HC Negatively Regulates MNR PPARγ

To figure out whether Inc-HC interacts with MNRs and its potential role in hepatocytic FFAs and TG metabolism, we analyzed the expression of MNRs in Inc-HC overexpressed and stable-knockdown cell lines, respectively named overLnc-HCCBRH and Lnc-HCshRCBRH. In overLnc-HCCBRH cells, mRNA expression of PPARγ and FXR, LXRxα, and Srebp1c expression in the CBRH-7919 cell model overtime (0, 3, 6, 12, 24, and 48 h). Data are expressed as means ± SEM. An unpaired t test was performed to determine the statistical significance. *p < 0.05; **p < 0.01; ***p < 0.001, as compared with, respectively, the vehicle group, the 0 h time point, or the NC group.

Inc-HC Controls miR-130b-3p Expression

The half-life of PPARγ mRNA was measured in Inc-HC interfered stable cell lines by the treatment of α-amanitin over 24 h to block RNA transcription by RNA polymerase II, using 18S rRNA as the normalizer control. Overexpression of Inc-HC reduced the half-life of the PPARγ transcript, while Inc-HC knockdown prolonged the half-life of the PPARγ transcript (Figure 3A). These results showed that Inc-HC negatively regulated PPARγ expression at the
As previously reported, lnc-HC regulates cholesterol catabolic genes Cyp7a1 and Abca1 through direct interaction with hnRNPA2B1 protein. In this study, RNA immunoprecipitation (RIP) with hnRNPA2B1 protein showed that hnRNPA2B1 could not bind the PPARγ transcript (Figure 3B). An RNA pulldown assay also doubly confirmed that, after the combination of lnc-HC and nucleoprotein (NP), there was no PPARγ transcript binding with the lnc-HC-NP complex (Figure 3C). In the RNA pulldown and hnRNPA2B1 RIP assay, Cyp7a1 was used as positive control, which has been verified to directly interact with hnRNPA2B1. To validate the direct interaction between lnc-HC and the PPARγ transcript, we performed a GFP-RIP assay following quantitative real-time PCR. The result indicates that lnc-HC could not enrich the PPARγ transcript, using immunoglobulin G (IgG) as NC and input as loading control (Figure 3D).

As we know, microRNAs (miRNAs) modulate gene expression at the post-transcriptional level. We used miRanda and TargetScan software to predict miRNA candidates regulating PPARγ expression, and predicted miRNAs include miR-130a-3p, miR-130b-3p, miR-301a-3p, and miR-301b-3p (Figure 3E). To confirm whether lnc-HC controls expression of these candidates, we conducted quantitative real-time PCR in lnc-HC-interfered CBRH-7919 and BRL3A cell lines. Results displayed that only miR-130b-3p expression was significantly increased with lnc-HC overexpression and decreased with lnc-HC knockdown (Figures 3F and S3A). Further, we checked the expression of primary (pri-), precursor (pre-), and mature miR-130b-3p in lnc-HC knockdown and NC control cell lines. With lnc-HC knockdown, pri-miR-130b was decreased (23% decrease), pre-miR-130b was expressed to a lower level (50% decrease), and miR-130b-3p was 42% decreased (Figure 3G), which suggests that miR-130b-3p is regulated...
Figure 3. lnc-HC Modulates miR-130b-3p Expression

(A) Quantitative real-time PCR analysis of PPARγ mRNA stability overtime (0, 6, 12, and 24 h) after α-amanitin treatment (50 μM) in lnc-HC stably overexpressed/knockdown cells. Data are expressed as means ± SEM. *p < 0.05, NC shR versus lnc-HC shR, two-way ANOVA. (B) RIP detection of the interaction between hnRNPA2B1 protein and lnc-HC or indicated mRNAs, including PPARγ, PPARα, Cyp7a1, and U6, by using anti-hnRNPA2B1 antibody (5 μg), with IgG (5 μg) used as antibody isotype control. (C) RNA pull-down assay to check the interaction among lnc-HC, nucleoprotein (NP), and indicated mRNAs, including PPARγ, PPARα, Cyp7a1, and U6. (D) RIP detection of the interaction between lnc-HC and mRNAs, including PPARγ and U6, by using anti-GFP antibody (5 μg), using IgG (5 μg) as the antibody isotype control and lnc-HC antisense (AS) as the negative control. (E) Bioinformatics prediction results of the candidate miRNAs targeting PPARγ 3′UTR and the binding sequence by using miRanda and TargetScan software. (F) Quantitative real-time PCR analysis of indicated miRNA expression in lnc-HC stably interfered CBRH-7919. (G) Quantitative real-time PCR analysis

(legend continued on next page)
by lnc-HC from the transcriptional level to the post-transcriptional level. Subsequently, we constructed a reporter gene vector of the miR-130b-3p promoter region as the pGL3-130b-3p-promoter and conducted a dual-luciferase reporter assay. Results showed that miR-130b-3p promoter activity increased with lnc-HC overexpression and decreased with lnc-HC knockdown (Figures 3H and S3B). As previously described, the second structure of lnc-HC contains a 5′ stem-loop, a central stem-loop, and a 3′ stem-loop.21 Here, a dual-luciferase reporter gene assay confirmed that the 5′ stem-loop (1–480 bp) of lnc-HC is critical for controlling miR-130b-3p promoter activity (Figures 3I and S3C).

miR-130b-3p Modulates PPARγ Expression

miR-130b-3p mimics and inhibitor (GenePharma, Shanghai, China) were used to check the regulation between miR-130b-3p and PPARγ expression. Mimics significantly reduced the PPARγ mRNA level in CBRH-7919 cells, which was originally upregulated by lnc-HC knockdown (Figure 4A). Consistent with the mRNA change, miR-130b-3p mimics also decreased PPARγ protein expression (Figure 4B). On the contrary, PPARγ was increased in CBRH-7919 cells after the treatment of miR-130b-3p inhibitor, at both the mRNA and protein levels (Figures 4C and 4D). Similarly, miR-130b-3p mimics strongly suppressed PPARγ expression, and miR-130b-3p inhibitor stabilized PPARγ expression in BRL3A cells (Figures 4A and 4B). We further constructed the reporter gene vectors pPPARγ-3′ UTR-wild and pPPARγ-3′ UTR-mut (Figure 4E). After transient co-transfection of CBRH-7919 cells with miR-130b-3p mimics/miRNC (NC mimic) or miR-130b-3p inhibitor/miRNC (NC inhibitor) and reporter gene vector pPPARγ-3′ UTR-wild/mut, cells were collected to perform a dual-luciferase reporter gene assay. In the pPPARγ-3′ UTR-wild transfection group, the relative luciferase activity was decreased with miR-130b-3p mimic treatment, while it was increased with inhibitor treatment. In addition, these changes were faded in the pPPARγ-3′ UTR-mut transfection group (Figure 4F). Thus, we demonstrated that miR-130b-3p decreases PPARγ expression through binding the PPARγ 3′ UTR. Combined with evidence that lnc-HC positively regulates miR-130b-3p expression, it is indicated that lnc-HC controls PPARγ expression through the post-transcriptional modulator miR-130b-3p.

lnc-HC/miR-130b-3p/PPARγ Regulates Hepatocytic Lipid Metabolism

After clarifying the negative regulation of lnc-HC on PPARγ expression, we further examined the effect of lnc-HC on hepatocytic lipid accumulation. Oil red O staining results showed that lnc-HC knockdown could increase the content of cellular lipid droplets after FFA treatment as compared with the group receiving NGSiR (NC shRNA [short hairpin RNA]) transfection plus FFA treatment (Figures 5A and 5B).
and 5B). Consistently, the concentration of intracellular FFAs and TGs was further increased in \( \text{lnc-} \text{HCshR} \) CBRH cells as compared with \( \text{N} \text{CshR} \) CBRH cells (Figures 5C and 5D). It seems contradictory that \( \text{lnc-} \text{HC} \) expression was upregulated in hepatocytes with FFA treatment, but \( \text{lnc-} \text{HC} \) knockdown increased lipid accumulation. Thus, we analyzed \( \text{lnc-} \text{HC} \) and \( \text{PPAR}_\gamma \) expression in \( \text{lnc-} \text{HC} \) stably interfered cells with vehicle and FFA treatment. Quantitative real-time PCR results showed that \( \text{PPAR}_\gamma \) was upregulated with FFA treatment, while its increase was much higher with the decrease of \( \text{lnc-} \text{HC} \) in \( \text{lnc-} \text{HCshR} \) CBRH-7919 cells; and \( \text{miR-130b-3p} \) expression was consistent with \( \text{lnc-} \text{HC} \) (Figure 5E). These results suggest that excessive FFA treatment increases \( \text{lnc-} \text{HC} \) and \( \text{PPAR}_\gamma \) expression; without \( \text{lnc-} \text{HC} \) upregulation, \( \text{PPAR}_\gamma \) could increase to a higher level and lead to excessive accumulation of lipid droplets in hepatocytes.

Next, we verified the effect of the \( \text{lnc-} \text{HC/miR-130b-3p/PPAR}_\gamma \) pathway on hepatic lipid accumulation. We respectively transfected \( \text{over} \text{lnc-} \text{HC} \) CBRH cells with \( \text{miR-130b-3p} \) inhibitor and transfected \( \text{lnc-} \text{HCshR} \) CBRH cells with \( \text{miR-130b-3p} \) mimics for 24 h and then treated cells with FFAs for 12 h. Oil red O staining showed that lipid droplet content was reduced with \( \text{lnc-} \text{HC} \) overexpression, while it increased with the transfection of \( \text{miR-130b-3p} \) inhibitor in \( \text{over} \text{lnc-} \text{HC} \) CBRH cells (Figures 5F and 5G). On the contrary, hepatic lipid accumulation was clearly increased with \( \text{lnc-} \text{HC} \) knockdown, while it decreased with the treatment of \( \text{miR-130b-3p} \) mimics in the \( \text{lnc-} \text{HCshR} \) CBRH cell line (Figures 5H and 5I). These results strongly indicated that the \( \text{lnc-} \text{HC/miR-130b-3p/PPAR}_\gamma \) pathway suppresses hepatic lipid accumulation in \( \text{in vitro} \).

**The Suppression of \( \text{lnc-} \text{HC} \) Aggravates Hepatic Lipid Disorder**

Next, we checked the \( \text{in vivo} \) effect of \( \text{lnc-} \text{HC} \) in hepatic lipid metabolism. Previously, E3 rats were treated with a high-fat, high-cholesterol diet (HCD; 10% fat, 4% cholesterol and 2% sodium cholate) for 8 weeks to induce a hepatic lipid disorder condition.\(^{21} \) H&E staining results showed that there was no macroscopic lipid droplet accumulation in the livers of the HCD and the HCD-plus-NCshR groups, but visible hepatic steatosis was already developed in the HCD-plus-\( \text{lnc-} \text{HCshR} \) group (Figure 6A). Serum lipid profiles were changed, TG and low-density lipoprotein-cholesterol (LDL-C) were significantly increased in the HCD group, and high-density lipoprotein-cholesterol (HDL-C) was decreased in the HCD group as compared with the control group; \( \text{lnc-} \text{HC} \) knockdown by using shRNA could improve serum parameters such as TG and HDL-C, but no effect was evident on serum LDL-C (Figure 6B). In addition, compared with the control group, the other three groups all showed higher liver/body weight ratios and significant increase of body weight gain (Figures 6C and 6D). Here, we focused on the hepatic metabolism changes with the variation of \( \text{lnc-} \text{HC} \) expression. TG concentration in rat livers was significantly increased in the HCD group as compared with the control group while further increased with \( \text{lnc-} \text{HC} \) knockdown by intravenous (i.v.) injection of \( \text{lnc-} \text{HC} \) shRNA into the HCD rat model as compared with the NC shR-plus-HCD group (Figure 6E). Meanwhile, we also checked the important gene expression in model livers. As previously shown, hepatic \( \text{lnc-} \text{HC} \) expression was increased in the HCD group and significantly suppressed to normal level in the \( \text{lnc-} \text{HC shR-plus-HCD} \) group (Figure 6F). \( \text{miR-} \text{130b-3p} \) expression was completely synchronized with \( \text{lnc-} \text{HC} \) (Figure 6G). In addition, \( \text{PPAR}_\gamma \) expression was significantly increased in the HCD group, as compared with the NC group, and its increase was higher in the \( \text{lnc-} \text{HC shR-plus-HCD} \) group at both the mRNA and protein levels (Figures 6H and 6I). Meanwhile, the overlapping gene \( \text{Sklka15} \) of \( \text{lnc-} \text{HC} \) significantly decreased with HCD treatment \( \text{in vivo} \) (Figure S5). These results also verified that \( \text{lnc-} \text{HC} \) positively regulates \( \text{miR-130b-3p} \) and that the \( \text{lnc-} \text{HC/miR-130b-3p} \) pathway negatively controls \( \text{PPAR}_\gamma \) expression. Furthermore, \( \text{lnc-} \text{HC/miR-130b-3p/PPAR}_\gamma \) delicately modulates hepatocytic lipid accumulation and might be a therapeutic target of NAFLD.

**DISCUSSION**

Aberrant lipogenesis and excessive accumulation of lipid droplets in liver indicate the pathogenesis of lipid metabolism disorders and NAFLD and is closely associated with diabetes, obesity, and atherosclerosis. However, the regulatory mechanism that controls hepatic lipogenesis remains uncertain. An approved therapeutic regime is lacking due to the complexity of NAFLD pathogenesis. Some existing studies have suggested that lncRNAs are emerging as an important modulator in almost all the biological processes, which includes hepatic lipid metabolism.\(^{22} - ^{27} \) In this study, we first identified \( \text{PPAR}_\gamma \) as the most prominent target of \( \text{lnc-} \text{HC} \) in hepatocytic lipid metabolism. Further study elucidates that \( \text{lnc-} \text{HC} \) plays a repressive role in hepatic lipid accumulation. By quantitative real-time PCR measurement of lipid MNR expression in \( \text{lnc-} \text{HC} \) overexpression and knockdown cells, \( \text{PPAR}_\gamma \) was identified as the most prominent target of \( \text{lnc-} \text{HC} \) in hepatocytic lipid metabolism. Both \( \text{in vivo} \) and \( \text{in vitro} \) results elucidate that \( \text{lnc-} \text{HC} \) negatively regulates \( \text{PPAR}_\gamma \) expression through \( \text{miR-130b-3p} \). Mechanically, there have been three critical regulatory patterns for lncRNAs in regulating gene expression. First, lncRNA directly interacts with specific protein as an lncRNA-protein complex, and they cooperatively control target gene expression.\(^{28} - ^{29} \) Second, lncRNAs directly bind target mRNA and regulate mRNA stability.\(^{30} \) Third, lncRNA and mRNA share the same recognition elements of miRNAs and increase the complexity of the miRNA regulation network, named the competitive endogenous pattern (ceRNA).\(^{31} - ^{32} \) We have reported that \( \text{lnc-} \text{HC} \) reduces the mRNA stability of \( \text{Cyp7a1} \) and \( \text{Abca1} \) through directly interacting with the co-regulator \( \text{hnRNPA2B1} \).\(^{21} \) Here, however, we have verified that \( \text{lnc-} \text{HC} \) regulates \( \text{PPAR}_\gamma \) does not through \( \text{lnc-} \text{HC-protein} \) or \( \text{lnc-} \text{HC}-\text{mRNA} \) patterns after RNA pulldown and RIP assay. In turn, \( \text{lnc-} \text{HC} \) regulates \( \text{PPAR}_\gamma \) expression through \( \text{miR-130b-3p} \), which is different from the three classical regulation patterns described earlier. \( \text{lnc-} \text{HC} \) controlling \( \text{miR-130b-3p} \) expression is involved in the level of transcription as well as the processing of miRNA precursors. As we known, the upstream signal regulation of miRNA expression is complicated. Further studies need to be done to figure out the detailed regulation between \( \text{lnc-} \text{HC} \) and \( \text{miR-130b-3p} \), which will help us understand not only the lncRNAs’ function but also miRNAs’ processing.
Figure 5. Inc-HC Negatively Regulates Hepatocytic Lipid Accumulation

(A and B) CBRH-7919, NCshR, and Inc-HCshR CBRH cells were treated with FFAs for 12 h, and the cellular lipid droplets in each group were (A) analyzed by oil red O staining and (B) measured by color density scanning using ImageJ. (C and D) The concentration of (C) intracellular FFAs and (D) TGs were detected. (E) Quantitative real-time PCR analysis of Inc-HC, PPARγ, and indicated miRNA expression in Inc-HC stably knocked down cells with vehicle or FFA treatment for 12 h. (F) OverInc-HC CBRH cells were transfected with NC inhibitor (iNC, 100 nM) or miR-130b-3p inhibitor (100 nM) for 24 h and then treated with FFAs for 12 h. Oil red O staining was used to detect the lipid droplet formation in each group. (G) The lipid droplet formation was measured in Inc-HCshR CBRH cells under the transfection with NC mimics (mNC, 50 nM) or miR-130b-3p mimics (50 nM) for 24 h and then treated with FFAs for 12 h. (H) Quantification histograms of the relative lipid droplet content in the indicated cells by using ImageJ. (I) Data are expressed as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001, as compared, respectively, with the NC group. #p < 0.05; ##p < 0.01; ###p < 0.001. An unpaired t test was performed to determine the statistical significance.
Figure 6. Inc-HC/miR-130b-3p Pathway Regulates Liver Lipid Metabolism

(A) H&E-stained liver sections with the HCD-induced lipid metabolism disorder E3 rat model. (B) At the end of the HCD-induced hyperlipidemia model (E3 rats, 8-week induction), serum concentrations of TG, HDL-C, and LDL-C were measured. (C and D) The liver/body weight ratio (C) and body weight gain (D) at the 8th week. (E) The hepatic TG concentration. (F and G) Quantitative real-time PCR analysis of Inc-HC expression (F) and miR-130b-3p and miR-130a-3p expression (G) in rat livers. (H and I) Quantitative real-time PCR analysis (H) and western blotting detection (I) of PPARγ expression in model livers. Data are expressed as means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. An unpaired t test was performed to determine the statistical significance.
Our previous findings declared that lnc-HC negatively regulates hepatic cholesterol catabolism. In an 8-week high-fat HCD-induced hepatic lipid disorder model, serum concentrations of TG and HDL-C were significantly improved with lnc-HC suppression, which indicates a therapy potential of lnc-HC knockdown in body lipid metabolism disorder. Regarding model liver, lnc-HC suppression significantly increased TG concentration and vascular-like steatosis phenotype. These parameters point out the multifunction of lnc-HC in systemic lipid metabolism as a good effect in dyslipidemia but as side effects in hepatic lipid metabolism. In vitro, the knockdown of lnc-HC significantly upregulates FFA uptake genes Fatp-1 and aP2 and TG synthesis genes Acc2, Acs1l, and Dgat2, while it has little effect on hepatocytic very low-density lipoprotein (VLDL) formation genes Dgat1 and Mttp. In vivo, lnc-HC has little effect on serum LDL-C concentration, which is derived mostly from TG-enriched VLDL-C. These results could explain the hepatic TG increase and serum TG decrease. In vivo, lnc-HC also negatively regulates mitochondrial FFA β-oxidation gene Cpt-1a, suggesting that its potential role in FFA consumption and utilization needs to be studied. Combined with the lipid accumulation test, we found that the comprehensive effect of lnc-HC is promoting FFA uptake, TG synthesis, and lipid droplet accumulation in cells. In vivo experiments showed that lnc-HC knockdown could aggregate lipid metabolism disorder in rat liver as vascular-like hepatic steatosis, which strongly indicates the protective role of lnc-HC in liver from excessive lipid droplet accumulation.

The exact signal that triggers lnc-HC expression is not measured in this study, but our previous work has reported that LXR agonist T0901317 could induce lnc-HC. Thus, LXRs might provide an upstream signal that drives hepatic lipogenesis and lnc-HC overexpression. Here, lnc-HC represses hepatocytic lipid droplet accumulation through negative regulation of PPARγ expression. It seems contradictory that FFA induces lnc-HC expression in CBRH-7919 and BRL3A cells, but lnc-HC overexpression suppresses hepatocytic lipid droplet formation, and knockdown lnc-HC expression increases hepatocytic lipid accumulation and liver TG concentration. Focused on this question, our quantitative real-time PCR results showed that PPARγ was upregulated in FFA-treated cells and HCD-treated rat livers, while it increased much higher with the decrease of lnc-HC, and that miR-130b-3p expression was consistent with lnc-HC. Hence, it is suggested that PPARγ could be upregulated much higher and lead to hepatocytic lipid overaccumulation without lnc-HC upregulation. This delicate modulation is consistent with the fine-tuning regulation pattern of lncRNAs and miRNAs. Here, our findings support a protection role of lnc-HC in preventing excessive lipogenesis, lipid accumulation, and NAFLD phenotype within the liver.

Taken together, we further identify the function and molecular mechanism of lnc-HC in inhibiting the accumulation of lipid droplets in liver through negatively regulating PPARγ expression. Under FFA treatment, the upregulated lnc-HC protects hepatocytes from excessively overexpressing PPARγ and helps avoid excessive formation of lipid droplets and lipid accumulation in liver. lnc-HC contributes hepatic lipid homeostasis through a versatile and complex mechanism, and these results may supply more detailed and integrated information to open new therapeutic strategies for the prevention and treatment of NAFLD.

**MATERIALS AND METHODS**

**Animals**

The animal experiments were approved by the Institutional Animal Ethics Committee of Xi’an Jiaotong University School of Medicine (permission ID: XJ2006Y039; Xi’an Jiaotong University, Xi’an, China), and were performed in accordance with the European Communities Council Directive 2010/63/EU for the protection of animals used for scientific purposes.

E3 rats originated from Lund University (Lund, Sweden) and were maintained in 12-h/12-h light/dark cycles under specific pathogen-free conditions. As previously described, 27-31 27 E3 rats (8–12 weeks old) were randomly divided into 4 groups, including a control group (n = 6), a high-fat HCD group (n = 7), an HCD and NC shRNA i.v. injection group (HCD, NCshR; n = 7), and an HCD and lnc-HC shRNA i.v. injection group (HCD, lnc-HCSH; n = 7). After being fed for 8 weeks with a normal diet or an HCD diet (10% fat, 4% cholesterol, and 2% sodium cholate), rats were euthanized by intraperitoneal (i.p.) injection of pentobarbital sodium (30 mg/kg) for blood collection, liver collection, and subsequent measurements.

**Cell Culture**

CBRH-7919 cells were maintained in RPMI-1640 medium (Hyclone Laboratories, South Logan, UT, USA) containing 10% FBS (GIBCO, Gaithersburg, MD, USA). BRL3A cells were maintained in DMEM (GIBCO, Gaithersburg, MD, USA) containing 10% FBS (GIBCO, Gaithersburg, MD, USA). For lipid droplet formation and oil red O staining, CBRH-7919 cells and BRL3A cells were plated in a 12-well plate. After 24 h, cells were treated with mixed FFAs (1 mM; PA-OA, 1:2 in RPMI-1640 medium containing 2% FBS) for the indicated time. Then, oil red O staining was conducted to determine the cellular lipid droplet formation. Treated cells were visualized using microscopy (Olympus, Tokyo, Japan). TG and FFA levels of cells and rat livers were measured, respectively, by using the Total Triglyceride Assay Kit (Nanjing Jiancheng, Nanjing, China) or the Non-esterified Free Fatty Acids Assay Kit (Nanjing Jiancheng, Nanjing, China), following the manufacturer’s instructions.

**lnc-HC and Slc25a15 Gene Overexpression and Knockdown**

Expression vector construction was as previously reported. Briefly, lnc-HC DNA was obtained by PCR with PrimeSTAR GXL DNA Polymerase (Takara Bio, Beijing, China) and inserted into the pcDNA3.1(+) vector between Hind III and EcoR I sites, named pcDNA3.1-lnc-HC. The Slc25a15 coding region was obtained by PCR and inserted into the pcDNA3.1(+) vector between Bam H I and Xho I, named pcDNA3.1-Slc25a15. Information on the primers is depicted in Table S1. The shRNAs for lnc-HC or Slc25a15 gene knockdown were ordered and synthesized by GenePharma. The target sequences for lnc-HC and Slc25a15 gene knockdown assay are depicted in Table S2. After a 24-h transfection...
of these recombinant plasmids in CBRH-7919 cells by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer’s instructions, G418 was added into cell culture medium at 500 µg/mL to select the monoclonal stable cell lines respectively named pPPARγ-CBRH, pSL-MS2-GFP-CBRH, and U6-CBRH. In Inc-HC interfered stable cells, small interfering RNAs (siRNAs) of PPARγ were used to check the regulation of Inc-HC on the PPARγ signaling pathway. The sequence information of PPARγ siRNAs is depicted in Table S2. In BRL3A cells, recombinant plasmids were transfected into cells by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) for 24 h for RNA isolation and 48 h for protein isolation.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

RNA from cells or rat liver tissues was extracted by using TRIzol Reagent (Invitrogen, Waltham, MA, USA). RNA concentration and quality were detected by NanoDrop. cDNAs for mRNA expression detection were reverse transcribed with 5 µg total RNA per sample by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and miRNA cDNAs were transcribed with 2 µg total RNA per sample by using the Mir-X miRNA qRT-PCR SYBR Kit (Clontech Laboratories, Mountain View, CA, USA). Quantitative real-time PCR was performed by using the SYBR Green qPCR Master Mix (Clontech, Mountain View, CA, USA) in the Agilent Mx3005P and Bio-Rad iQ5 systems. The detection program of gene expression with cDNA transcribed from mRNA is: 10 min at 95°C, 40 cycles of 10 s at 95°C, 10 s at annealing temperature, and 30 s at 72°C. The program of miRNA cDNA detection is: 10 min at 95°C, 40 cycles of 10 s at 95°C, and 30 s at 60°C. Melting curves were performed to check quantitative real-time PCR products. Data were normalized by β-actin for mRNA expression and by U6 for miRNAs. The gene primer information for quantitative real-time PCR is depicted in Table S3, mRNA primer information for quantitative real-time PCR is depicted in Table S4.

Western Blotting

Protein was isolated from cells and rat model livers by using cell lysis buffer (Beyotime Biotechnology, Shanghai, China). Protein samples were separated by 10% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) according to the standard process. Gene expression at the protein level was detected by using rabbit anti-PPARγ antibody (Abcam, Cambridge, MA, USA; 1:1,000), rabbit anti-Slc25a15 antibody (Abcam, Cambridge, MA, USA; 1:1,000) or rabbit anti-β-actin antibody (Santa Cruz Biotechnology, Shanghai, China; 1:500).

RNA Immunoprecipitation

For detecting the interaction between hnRNPA2B1 and candidate RNAs, RIP was performed as previously described.21,26,33 Briefly, the cell nucleus was obtained by using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China). Nuclear extract was lysed with polysome lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM HEPES [pH 7.0], 0.5% NP-40, and 1 mM DTT, with added complete protease inhibitors [Roche, Basel, Switzerland] and RNase inhibitor [Ambion, Austin, TX, USA] before use). Rabbit anti-hnRNPA2B1 antibody (Abcam, Cambridge, UK) or rabbit isotype IgG per 5 µg was used in each respective RIP reaction. Binding RNAs were isolated by phenol-chloroform-isooamyl alcohol for quantitative real-time PCR assay.

To detect the direct interaction between Inc-HC and candidate mRNAs, we constructed recombinant plasmids first. The Inc-HC DNA was obtained by PCR with PrimeSTAR GXL DNA Polymerase (Takara Bio, Beijing, China) and inserted forward or reversely into pcDNA3.1 (+) vector between Hind III and EcoR I sites, respectively named pcDNA3.1-Inc-HC(sense) and pcDNA3.1-Inc-HC(antisense). Then, pSL-MS2-12X (Addgene, USA), pcDNA3.1-Inc-HC(sense), and pcDNA3.1-Inc-HC(antisense) were double digested with EcoR I and Xho I. The MS2-12X fragment, the digestion product of pSL-MS2-12X, was inserted into pcDNA3.1-Inc-HC(sense) or pcDNA3.1-Inc-HC(antisense), respectively named pcDNA3.1-MS2-Inc-HC(sense) and pcDNA3.1-MS2-Inc-HC(antisense). These vectors were used to determine the interaction between Inc-HC and candidate mRNAs by RIP assay. CBRH-7919 cells were co-transfected with pMS2-GFP (Addgene, Watertown, MA, USA) and pcDNA3.1-MS2-Inc-HC(sense) or pcDNA3.1-MS2-Inc-HC(antisense). After 48 h of transfection, cells were harvested to perform a RIP assay by using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Cleveland, OH, USA) according to the manufacturer’s instructions. Rabbit anti-GFP antibody (Abcam, Cambridge, UK) or rabbit isotype IgG (Beyotime Biotechnology, Shanghai, China) per 5 µg were used in each respective RIP reaction system.34

RNA Pulldown

For detecting the interaction among Inc-HC, nucleus protein, and target mRNAs, RNA pulldown was performed by using the Pierce Magnetic RNA-Protein Pull-Down Kit (Pierce, Waltham, MA, USA). Briefly, biotin-labeled RNAs were transcribed in vitro by using Biotin RNA Labeling Mix (Roche, Basel, Switzerland) and T7 RNA polymerase (Ambion, Austin, TX, USA), and purified by phenol-chloroform-isoamyl alcohol. After the binding between folded biotin-Inc-HC (sense or antisense) and nucleus protein, folded total RNA (~250 µg) of CBRH-7919 cells was added into purified beads. After incubation and bead washing, the RNA component was isolated by phenol-chloroform-isoamyl alcohol for quantitative real-time PCR assay.

Dual-Luciferase Reporter Gene Assay

The recombinant vectors of PPARγ 3’UTR (wild and mut) for checking interaction between miRNA candidates and PPARγ by dual-luciferase reporter gene assay were constructed by GeneChem (Shanghai, China) and, respectively, named pPPARγ-3’UTR-wild and pPPARγ-3’UTR-mut. The DNA fragments of PPARγ 3’UTR-wild and PPARγ 3’UTR-mut are listed in Table S1. CBRH-7919 cells were seeded into a 48-well plate. CBRH-7919 cells were co-transfected with pPPARγ-3’UTR-wild (100 ng) or pPPARγ-3’UTR-mut (100 ng) and miRNA
small molecules (100 nM), such as mNCs, miR-130b-3p mimics, iNCs, or miR-130b-3p inhibitors (purchased from GenePharma, Shanghai, China) by Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA). After 24 h of transfection, the relative luciferase activity of each group was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), according to the manufacturer’s instructions.

The promoter regions of miR-130b-3p and miR-130a-3p were generated by PCR. The primer information is depicted in Table S1. They were cloned into pGL3-basic vectors between KpnI and Nhe I sites named pGL3-130b-3p-promoter and pGL3-130a-3p-promoter, respectively. For detecting the promoter activity of miR-130a-3p and miR-130b-3p, pGL3-promoter/pRL (90:10 ng per well) was co-transfected into CBRH-7919 or BRL3A cells (in a 48-well plate) for 24 h. pGL3-basic/pRL (90:10 ng per well) was used as NC. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), following the manufacturer’s instructions.

Statistical Analysis
Quantitative data were expressed as means ± SEM. The statistical analysis of difference between groups was performed by unpaired Student’s t test. A two-way ANOVA was used to analyze the differences of mRNA stability after α-amanitin treatment in indicated cell lines. p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Study design: X.L. and S.L. Analysis and interpretation of data: S.L. and X.L. Drafting of the manuscript: X.L. Critical revision of the manuscript: D.L., X. Yan, and X. Yang. Necessary technical guides: D.L., X. Yan, and X. Yang. Obtained funding: X.L. Performance of experiments: X.L., N.W., Q.C., and L.W. Preparation and assistant work: Yue L., X.D., C.W., L.F., Yazhao L., E.K.O., M.S., and Q.N. Necessary technical guides: D.L., X. Yan, and X. Yang.

CONFLICT OF INTERESTS
The authors declare no competing interests.

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