Long Noncoding RNA HULC Modulates Abnormal Lipid Metabolism in Hepatoma Cells through an miR-9-Mediated RXRA Signaling Pathway

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Abstract

HULC is a long noncoding RNA overexpressed in hepatocellular carcinoma (HCC), but its functional contributions in this setting have not been determined. In this study, we explored the hypothesis that HULC contributes to malignant development by supporting abnormal lipid metabolism in hepatoma cells. HULC modulated the deregulation of lipid metabolism in HCC by activating the acetyl-CoA synthetase subunit ACSL1. Immunohistochemical analysis of tissue microarrays revealed that approximately 77% (180/233) of HCC tissues were positive for ACSL1. Moreover, HULC mRNA levels correlated positively with ACSL1 levels in 60 HCC cases according to real-time PCR analysis. Mechanistic investigations showed that HULC upregulated the transcriptional factor PPARa, which activated the ACSL1 promoter in hepatoma cells. HULC also suppressed miR-9 targeting of PPARa mRNA by eliciting methylation of CpG islands in the miR-9 promoter. We documented the ability of HULC to promote lipogenesis, thereby stimulating accumulation of intracellular triglycerides and cholesterol in vitro and in vivo. Strikingly, ACSL1 overexpression that generates cholesterol was sufficient to enhance the proliferation of hepatoma cells. Further, cholesterol addition was sufficient to upregulate HULC expression through a positive feedback loop involving the retinoid receptor RXRA, which activated the HULC promoter. Overall, we concluded that HULC functions as an oncogene in hepatoma cells, acting mechanistically by deregulating lipid metabolism through a signaling pathway involving miR-9, PPARa, and ACSL1 that is reinforced by a feed-forward pathway involving cholesterol and RXRA to drive HULC signaling. Cancer Res; 75(5); 846–57. ©2015 AACR.

Introduction

Growing evidence indicates that many noncoding regulatory elements are transcribed into noncoding RNAs (ncRNA; refs. 1, 2). Several types of ncRNAs are regarded as regulatory RNAs that possess orchestrated functions involved in the control of genome dynamics, cell biology, and developmental programming (3). NcRNA is habitually divided into two groups on the basis of transcript size: long ncRNA (lncRNA, >200 nt long) and small ncRNA (4). A spot of characterized human lncRNAs has been associated with a spectrum of biologic functions, and the disruption of these functions plays a critical role in the development of cancer (5). Highly upregulated in liver cancer (HULC) is the first identified lncRNA specifically overexpressed in hepatocellular carcinoma (HCC; ref. 6). HULC is transactivated by CREB and sequesters miR-372 by acting as a sponge (7), and insulin-like growth factors 2 mRNA-binding proteins (IGF2BP) are able to govern the expression of HULC (8). Previously, our group reported that hepatitis B virus X protein (HBx)–elevated HULC could accelerate the growth of hepatoma cells by downregulating p18 (9). However, the role of HULC in abnormal lipid metabolism remains poorly understood.

HCC is the fifth-most common cancer worldwide and the third largest cause of cancer death globally (10). Recently, mounting clinical and epidemiologic studies have reported that high-risk cancer is linked to metabolic syndromes, such as obesity, type II diabetes, and atherosclerosis (11). Lipids, which represent a diverse group of water-insoluble molecules, play essential roles in these processes. Meanwhile, high rates of lipid uptake and de novo lipid synthesis are frequently exhibited by cancer cells. For example, various tumors and their precursor lesions undergo exacerbated endogenous fatty acid biosynthesis irrespective of the levels of extracellular lipids (12). The changes in lipid metabolism can alter numerous cellular processes, including proliferation, motility, and tumorigenesis. As a central organ of energy metabolism, the liver synthesizes most plasma apolipoproteins, endogenous lipids, and lipoproteins. Thus, the advent of HCC is accompanied by metabolic reprogramming, which is reflected in changes in gene expression and miRNA profiles as well as altered levels of circulating proteins and small metabolites (13). The Akt/mTOR pathway and insulin signaling have been reported to contribute to the deregulation of lipid metabolism in hepatoma cells thus far (14, 15).
Acyl-CoA synthetase long-chain family members (ACSL) catalyze the initial step in cellular long-chain fatty acid metabolism in mammals (16). There are five members in this family. Among them, ACSL1 is one of the major isoforms, with high levels in the liver, and can be regulated by the transcriptional factor peroxisome proliferator–activated receptor alpha (PPARA; refs. 17, 18). Some studies have reported that the overexpression of ACSL1 increases the uptake of fatty acids in hepatoma cells (19). However, whether ACSL1 contributes to the development of HCC has been ill-documented. The retinoid X receptors (RXR) were identified in 1990 as orphan receptors that exhibited diverse transcriptional responses (20). RXRs play a vital role in the nuclear receptor superfamily, forming heterodimers with many other family members; as a result, RXRs are implicated in the control of various physiologic processes (21). RXRA is a member of the RXR family that can be activated by sterol (22). In humans, cholesterol homeostasis is maintained by the precise interactions between intestinal uptake, de novo synthesis, hepatic output, and fecal disposal (23), and excessive or deficient cholesterol can result in pathophysiological sequelae (24). Secreted apoA-I binding protein (AIBP) positively regulates cholesterol efflux from endothelial cells, and effective cholesterol efflux is critical for proper angiogenesis (25). In addition, the primary metabolite of cholesterol, 27-hydroxycholesterol (27HC), contributes to estrogen receptor–dependent growth and liver X receptor–dependent metastasis in mouse models of breast cancer (26), and cholesteryl ester accumulation induced by PTEN loss and PI3K/AKT activation underlies human prostate cancer aggressiveness (27). However, the general mechanism responsible for aberrant lipid metabolism during the development of HCC is not well understood.

In the present study, we investigated the role of lncRNAs in the abnormal lipid metabolism of HCC. Intriguingly, our data demonstrate that the IncRNA HULC facilitates the deregulation of lipid metabolism through miR-9/PPARA/ACSL1/cholesterol/RXRA/HULC signaling. Thus, our finding provides new insights into the mechanism of aberrant lipid metabolism in HCC.

Materials and Methods

Patient samples

Sixty HCC tissue samples and their corresponding adjacent nontumorous liver tissues were obtained from Tianjin First Center Hospital and Tianjin Tumor Hospital (Tianjin, China) after surgical resection. Fifty-five of 60 patients had a history of hepatitis B virus infection. Written consent approving the use of tissue samples for research purposes was obtained from patients. The information of patients with HCC is presented in Supplementary Table S1. The study protocol was approved by the Institute Research Ethics Committee at Nankai University.

Cell lines and cell culture

The human hepatoma H7402 cell line, human immortalized liver L-02 cell line, and Chang liver cell line were cultured in RPMI-1640 medium (Gibco). The human hepatoma cell lines, Huh7 (obtained from Shanghai Institutes for Biological Sciences), HepG2, and HepG2.2.15 (a hepatoma HepG2 cell line with integrated full-length hepatitis B virus DNA), and human kidney epithelial (HEK) 293T cells were maintained in DMEM (Gibco). All cell lines were supplemented with heat-inactivated 10% FBS (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin and grown at 5% CO₂ and 37°C.

In vivo tumorigenicity assay

Nude mice were housed and treated according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We conducted animal transplantations according to the Declaration of Helsinki. Briefly, HepG2 (or Huh7) cells were harvested and resuspended at 2 × 10⁶ cells per mL in sterile PBS. Groups of 4-week-old male BALB/c athymic nude mice (Experiment Animal Center of Peking, China; each group, n = 6) were subcutaneously injected at the shoulder with 0.2 mL of the cell suspensions. According to the protocol (28), cholesterol (Solarbio) was subcutaneously injected into the appropriate mice once in proximity to the tumor after injection of 5 days. Group one, the control group, was injected with 100 μL aceto. Group two and three were experimental groups and were injected with 300 μmol/L/kg cholesterol in 100 μL aceto. All cells transplanted in mice in group three were pretreated with 100 nmol/L HULC siRNA. Tumor growth was measured beginning 5 days after injection of hepatoma cells. Tumor volume (V) was monitored by measuring the length (L) and width (W) of the tumors with calipers and was calculated using the formula (L × W²) × 0.5. After 25 days, tumor-bearing mice and controls were sacrificed, and the tumors were excised and measured.

Statistical analysis

Each experiment was repeated at least three times. Statistical significance was assessed by comparing mean values (6 SD) using the Student t test for independent groups as follow: *P < 0.05; **P < 0.01; ***P < 0.001 and not significant (NS). The Pearson correlation coefficient was used to determine the correlations among gene expression in tumorous tissues. ACSL1 expression in tumor tissues and matched adjacent nontumor tissues was compared using the Wilcoxon signed-rank test.

Results

HULC is positively correlated with ACSL1 in clinical HCC tissues and upregulates ACSL1 in hepatoma cells

To demonstrate the role of HULC in the deregulation of lipid metabolism in HCC, we examined the effects of HULC on several lipid metabolic enzymes in hepatoma cells. ACSL1 stood out as being noticeably upregulated by HULC (Supplementary Fig. S1A). Thus, we evaluated the expression of ACSL1 by immunohistochemical (IHC) staining in clinical HCC tissues using tissue microarrays and found that 77.3% (180/233) of HCC tissues were positive for ACSL1 compared with 12.5% (2/16) of peritumoral liver tissues, in which the expression of ACSL1 was stronger in HCC tissues than that in their peritumoral liver tissues (Fig. 1A). Moreover, quantitative real-time PCR (qRT-PCR) revealed that the mRNA levels of ACSL1 were higher in HCC tissues compared with their adjacent nontumorous liver tissues in 60 paired clinical HCC samples (Fig. 1B). ACSL1 has been reported to participate in the formation of triglycerides and cholesterol in the liver (29). Our data demonstrated that the increased triglycerides/cholesterol was accumulated in HCC tissues relative to their corresponding peritumoral tissues (Supplementary Fig. S1B). Furthermore, we observed that the levels of HULC were positively associated with those of ACSL1, triglycerides, and cholesterol in the aforementioned clinical samples (Fig. 1C). Moreover, we found that...
overexpression of HULC upregulated ACSL1 in HepG2 and Huh7 (or L-O2) cells at the mRNA and protein levels in a dose-dependent manner (Supplementary Fig. S1C; Fig. 1D and 1E; and Supplementary Fig. S1D). Similarly, depletion of HULC led to a decrease in ACSL1 levels in HepG2.2.15 cells expressing high levels of endogenous HULC (Supplementary Fig. S1E and Fig. 1F). Meanwhile, the transfection (or interference) efficiency of HULC (or HULC siRNA) was validated by qRT-PCR or RT-PCR analysis (Supplementary Fig. S1C–S1E and Fig. 1D–F). Strikingly, HULC was capable of enriching triglycerides and cholesterol in HepG2 cells in a dose-dependent manner (Supplementary Fig. S1F), but failed to increase their levels in the conditioned medium (Supplementary Fig. S1G). Taken together, these results show that HULC is positively associated with ACSL1 in clinical HCC tissues and upregulates ACSL1 in hepatoma cells.
HULC upregulates the transcriptional factor PPARA to activate ACSL1

Given that ACSL1 is activated by the transcriptional factor PPARA in the liver (17), we speculated that HULC might modulate ACSL1 through PPARA. Interestingly, qRT-PCR analysis demonstrated that the expression levels of HULC were positively associated with those of PPARA in the 60 clinical HCC samples (Fig. 2A). However, inhibition of PPARA expression abrogated the HULC-induced upregulation of ACSL1 in HepG2 cells (Fig. 2B), suggesting that PPARA is responsible for the upregulation of ACSL1 mediated by HULC. The efficiency of PPARA siRNA (or PPARA siRNA*) was validated in these cells (Supplementary Fig. S2A). Moreover, the overexpression of HULC was able to upregulate PPARA at mRNA and protein levels in HepG2 and Huh7 cells (or L-O2 cells) in a dose-dependent manner (Fig. 2C–E and Supplementary information; Supplementary Fig. S2B and S2C), whereas HULC siRNA reversed these effects in HepG2.2.15 cells (Fig. 2F and G). The transfection (or interference) efficiency of HULC (or HULC siRNA) was confirmed in these cells (Fig. 2C–G and Supplementary Fig. S2B and S2C). However, luciferase reporter gene assays showed that HULC failed to activate the promoter of PPARA in HepG2 cells (Supplementary Fig. S2D and S2E), implying that HULC might upregulate PPARA at the posttranscriptional step. Thus, we conclude that HULC activates ACSL1 by upregulating the transcription factor PPARA in hepatoma cells.

miR-9 inhibits the expression of PPARA by targeting the 3' UTR of PPARA

Next, we identified several miRNAs that could potentially bind to the 3' untranslated region (UTR) of PPARA using

Figure 2.

HULC upregulates the transcriptional factor PPARA to activate ACSL1. A, the correlation between HULC mRNA levels and PPARA mRNA levels was examined by qRT-PCR in 60 cases of clinical HCC tissues (P < 0.01; Pearson correlation coefficient, r = 0.8517). B, the effect of PPARA siRNA on HULC-enhanced ACSL1 was examined by Western blotting in HepG2 cells. The transfection efficiency of HULC and the interference efficiency of PPARA were detected by qRT-PCR and Western blotting, respectively. C–E, HepG2 and Huh7 cells were transfected with pcDNA3.1-HULC, and the mRNA (or protein) levels of PPARA were examined by RT-PCR (or Western blotting). The transfection efficiency of HULC was detected by RT-PCR (or qRT-PCR). F and G, HULC siRNA was transfected into HepG2.2.15 cells, and the mRNA (or protein) levels of PPARA were assessed by RT-PCR (or Western blotting). The interference efficiency of HULC was detected by RT-PCR (or qRT-PCR). Statistically significant differences are indicated: **, P < 0.01; Student t test.
TargetScan and microrna.org (http://www.targetscan.org, http://www.microrna.org/microrna/home.do). Because miR-9 has been reported to be downregulated in cancer (30–32), we focused our investigation on this miRNA. Three miR-9 binding sites in the 3′ UTR of PPARA mRNA were constructed (Fig. 3A and Supplementary Fig. S3A and S3B), and luciferase reporter assays revealed that miR-9 could directly bind to the conserved seed region of the PPARA 3′ UTR (position 7624-7631, pGL3-PPARA-7624; Fig. 3B), rather than the poorly conserved seed regions (position 5684-5690, position 4375-4381; Supplementary Fig. S3C and S3D). However, the PPARA 3′ UTR conserved seed region mutant (position 7624-7631, pGL3-PPARA-mut) failed to work in these cells (Fig. 3B). Conversely, anti–miR-9 increased the luciferase activities of pGL3-PPARA-7624 but failed to influence the mutant (Fig. 3C), suggesting that miR-9 is able to directly bind to the 3′ UTR of PPARA. These effects were also observed in 293T cells (Supplementary Fig. S3C–S3F). Furthermore, the overexpression of miR-9 suppressed the expression of PPARA in HepG2 cells in a dose-dependent manner (Fig. 3D), and the reverse outcome was obtained when the cells were treated with anti–miR-9 (Fig. 3E). Together, our data indicate that miR-9 suppresses the expression of PPARA by targeting its 3′ UTR.

HULC downregulates miR-9 through inducing methylation of CpG islands in its promoter

Next, we validated the anticorrelation between HULC and miR-9 in the 60 clinical HCC samples (Fig. 4A). Then, our data showed that the overexpression of HULC downregulated miR-9 in HepG2 cells in a dose-dependent manner (Fig. 4B). Interestingly, we found that treatment with 5-Aza-2′-deoxycytidine (Aza, a DNA methylation inhibitor) heightened the levels of miR-9 in HepG2 cells in a dose-dependent manner (Fig. 4C), suggesting that HULC might influence the epigenetic regulation of the miR-9 promoter (33, 34). Then, we examined the methylation status of miR-9 using both methylation-specific PCR (MSP) and bisulfite-sequencing analysis (BSP). As shown in Fig. 4D, miR-9 comprises three members, termed miR-9-1, miR-9-2 and miR-9-3. MSP assays revealed that the CpG sites of miR-9-1 (or miR-9-2, miR-9-3) were highly methylated following the overexpression of HULC in L-O2 (or Chang liver (Chang), HepG2, and H7402) cells. MSP assays further validated the above observations in L-O2 (or HepG2) cells (Fig. 4E and Supplementary Fig. S4A). Moreover, we confirmed these data in two pairs of clinical samples (Fig. 4F and Supplementary Fig. S4B). It has also been reported that DNA (cytosine-5-)-methyltransferase 1 (DNMT1), a methylase, is capable of regulating the expression of miRNAs through inducing
methylation of their CpG islands (35). Interestingly, we observed that HULC was able to upregulate DNMT1 in HepG2 cells (Supplementary Fig. S4C), hinting that HULC might induce methylation of CpG islands in the miR-9 promoter through upregulation of DNMT1. Therefore, we conclude that HULC inhibits the expression of miR-9 through eliciting methylation of CpG islands in the miR-9 promoter.

The product cholesterol of ACSL1 is able to upregulate HULC by activating RXRA in hepatoma cells

Given that the positive feedback loops in signaling pathways are involved in the progression of cancer, we evaluated whether HULC facilitates aberrant lipid metabolism in liver cancer via a feedback mechanism. Surprisingly, we found that the promoter activities of HULC were dose-dependently decreased in HepG2 cells after treatment with Triacsin C (an inhibitor of ACSL1) or ACSL1 siRNA, although this was not observed in L-O2 cells (Fig. 5A and Supplementary Fig. S5A). Meanwhile, we observed that the expression levels of HULC were also downregulated in HepG2 cells (Fig. 5B and Supplementary Fig. S5B), suggesting that a positive feedback loop involving HULC/miR-9/PPARA/ACSL1/HULC is established in hepatoma cells but not in normal liver cells. Next, we utilized acetone as the solvent to deliver triglyceride (or cholesterol) to the cells. Strikingly, we found that cholesterol was able to stimulate the activity of the HULC promoter in HepG2 (or Huh7) cells in a dose-dependent manner, although triglyceride failed to have an effect in these cells (Fig. 5C and D, Supplementary information, S5C and S5D). According to the report (22), we cloned the HULC...
promoter including the mutant in the RXRA-binding site (Fig. 5E). Intriguingly, the treatment with cholesterol failed to activate the above mutant in HepG2 cells (Fig. 5E), suggesting that RXRA may be implicated in the regulation of HULC mediated by cholesterol. Moreover, we verified that RXRA siRNA could abolish the cholesterol-increased HULC promoter activity (Fig. 5F). However, the treatment with cholesterol failed to influence the expression of RXRA (Supplementary Fig. S5E), suggesting that cholesterol, as a type of sterol, might be able to activate the HULC promoter by stimulating RXRA, rather than upregulating RXRA. Meanwhile, the efficiencies of ACSL1 siRNA (or ACSL1 siRNA') and RXRA siRNA were validated by Western blot analysis in these cells (Supplementary Fig. S5F and S5G). As a result, we conclude that the product cholesterol of ACSL1 upregulates HULC by activating RXRA in hepatoma cells.

HULC disrupts the lipid metabolism of hepatoma cells through miR-9/PPARA/ACSL1 signaling in vitro

Next, we investigated the effect of HULC on lipogenesis in hepatoma cells using Oil Red O staining. These results showed that the overexpression of HULC was able to accelerate lipogenesis in HepG2 and Huh7 cells, whereas ACSL1 siRNA (or miR-9, PPARA siRNA, Triacsin C) could block this event. Inversely, anti-miR-9 was capable of enhancing lipogenesis in the cells (Fig. 6A). In addition, we validated that the treatment with HULC siRNA (or ACSL1 siRNA, miR-9, PPARA siRNA, and Triacsin C)
could attenuate the lipogenesis in HepG2.2.15 cells. However, anti–miR-9 was able to rescue the HULC siRNA-repressed lipogenesis (Fig. 6B), and we obtained a similar effect of HULC on intracellular triglyceride and cholesterol in this system (Fig. 6C and D; Supplementary Fig. S6A and S6B). The role of ACSL1 in the growth of hepatoma cells remains enigmatic. In this study, we showed that the overexpression of ACSL1 could facilitate the proliferation of HepG2 and Huh7 cells, as demonstrated by MTT assays and cloning formation assays (Fig. 6E and F). Thus, we sought to evaluate whether the product cholesterol of ACSL1 may be responsible for the promotion of cell proliferation. As expected, MTT assays further corroborated that the treatment with cholesterol was able to promote the proliferation of hepatoma cells, which could be eliminated by HULC siRNA (Supplementary Fig. S6C), suggesting that cholesterol might enhance the proliferation of hepatoma cells by upregulating HULC. In addition, we assessed whether HULC affected other signaling pathways and factors involved in lipid metabolism in hepatoma cells, such as peroxisome proliferator-activated receptor alpha (PPARA), and the results were presented in Figure 6A and Fig. 6A, the effect of HULC (or anti–miR-9, miR-9, PPARA siRNA, ACSL1 siRNA, and Triacsin C) on lipogenesis was determined by Oil Red O staining in HepG2 (or Huh7) cells. B, the effect of HULC siRNA (or anti–miR-9, miR-9, PPARA siRNA, ACSL1 siRNA, and Triacsin C) on lipogenesis was determined by Oil Red O staining in HepG2.2.15 cells. C and D, the effect of HULC (or anti–miR-9, miR-9, PPARA siRNA, ACSL1 siRNA, and Triacsin C) on cellular triglycerides (or cholesterol) was measured in HepG2 cells using the tissue triglyceride assay kit (or tissue total cholesterol kit). E and F, the effect of ACSL1 on the proliferation of hepatoma cells was assessed by MTT assays and cloning formation assays in HepG2 and Huh7 cells, respectively. Statistically significant differences are indicated: *, \( P < 0.05 \); **, \( P < 0.01 \); NS, nonsignificant; Student t test.
as the AKT/mTOR pathway, SREBP1, SREBP2, chREBP, HMGCR, FASN, AGLY, SQS, and miR-122 (14, 36). However, we did not observe alterations in these factors at the levels of mRNA and protein in HepG2 and Huh7 cells (Supplementary Fig. S6D–S6G). Thus, we conclude that HULC contributes to aberrant lipid metabolism in hepatoma cells through miR-9/PPARA/ACSL1 signaling.

HULC-modulated abnormal lipid metabolism facilitates tumor growth in vivo

To better understand the role of HULC in abnormal lipid metabolism, we subcutaneously injected pretreated cells into 4-week-old BALB/c athymic nude mice. We confirmed that the levels of HULC and ACSL1 were preserved in the tumor tissues (Supplementary Fig. S7A). We observed that treatment with ACSL1 siRNA abolished the HULC1-accelerated proliferation of HepG2 (or Huh7) cells in mice (Fig. 7A and B and Supplementary Fig. S7B), further supporting the conclusion that ACSL1 is responsible for the promotion of tumor growth mediated by HULC. IHC staining further confirmed that the expression of Ki-67, a marker of proliferation, as well as BrdUrd incorporation in the tumor tissues was consistent with tumor growth among the different groups (Supplementary Fig. S7C). Interestingly, oil red O staining revealed that lipid droplets were increased in the tumor tissues overexpressing HULC, whereas silencing of ACSL1 reversed this effect (Fig. 7C and Supplementary Fig. S7C). The levels of triglycerides and cholesterol were consistent with the Oil Red O staining in the tumor tissues (Fig. 7D and E), suggesting that abnormal lipid metabolism contributes to the growth of hepatoma cells. To better evaluate the effect of

Figure 7. HULC-modulated abnormal lipid metabolism facilitates the tumor growth in vivo. A, photographs of dissected tumors from nude mice tumor transplanted with HepG2 (or Huh7) cells pretreated with pcDNA3.1, pcDNA3.1-HULC, or pcDNA3.1-HULC, and ACSL1 siRNA together. B, the average weight of tumors from experimental groups of nude mice. C, the levels of triglycerides were individually measured using a tissue triglyceride assay kit in the tumor tissues from each nude mouse transplanted with HepG2 cells. D, the levels of cholesterol were individually measured using a tissue total cholesterol kit in the tumor tissues from each nude mouse transplanted with HepG2 cells. E, the levels of triglycerides and cholesterol were individually measured using a tissue total cholesterol kit in the tumor tissues from each nude mouse transplanted with HepG2 cells. F, photographs of dissected tumors from nude mice transplanted with HepG2 (or Huh7) cells treated with cholesterol or cholesterol/HULC siRNA. G, the average weight of the tumors from the experimental groups of nude mice. Statistically significant differences are indicated: *P < 0.05; **P < 0.01; Student t test.
cholesterol on the expression of HULC in hepatoma cells in vivo, we injected supraphysiological cholesterol in proximity to the tumor tissues. As expected, the injection significantly increased the levels of tissue HULC (Supplementary Fig. S7D). Notably, we observed that the pretreatment with HULC siRNA remarkably abolished the cholesterol-increased growth of hepatoma cells (Fig. 7F and G and Supplementary Fig. S7E), suggesting that cholesterol is able to upregulate HULC in hepatoma cells. Together, we conclude that HULC-modulated abnormal lipid metabolism contributes to tumor growth, and this process requires the metabolic enzyme ACSL1 and its product cholesterol.

Discussion

LncRNAs play crucial roles in cancer (37), and we previously reported that HBs-enhanced HULC is able to promote the growth of hepatoma cells (9). Metabolism deregulation, exacerbated lipid biosynthesis, and accumulation in the development of cancer accelerate cell growth and transformation (38). In this study, we assessed whether HULC participates in abnormal lipid metabolism in HCC.

To better understand the roles of HULC in modulating lipid metabolism, we first measured the effect of HULC on lipid metabolic enzymes in hepatoma cells. Interestingly, ACSL1 drew our attention because ACSL1 and its intracellular products, such as triglycerides and cholesterol, were remarkably upregulated and increased by HULC expression. However, the levels of triglycerides and cholesterol in the conditioned medium were unchanged in HULC-transduced cells relative to controls, which may be the result of the ability of ACSL1 to inhibit cholesterol efflux (39). Moreover, we validated that the expression levels of HULC were positively correlated with those of ACSL1 and its products (40). Hence, we validated that the expression of HULC was negatively correlated with those of ACSL1 and its products in clinical HCC samples (Fig. 1 and Supplementary Fig. S1). Next, we explored the mechanism by which HULC activates ACSL1 in hepatoma cells. ACSL1 has been reported to be a classic target gene of PPARA in the liver (16). Accordingly, we observed that HULC modulated ACSL1 by upregulating PPARA (Fig. 2 and Supplementary Fig. S2). Furthermore, we determined that HULC could increase PPARA expression by downregulating the ability of miR-9 to target the PPARA 3′UTR at the posttranscriptional level, rather than by activating the transcription of PPARA directly (Fig. 3 and Supplementary Fig. S3). Numerous studies have noted that the expression of miR-9 is regulated by CpG island methylation, and lncRNAs are able to modulate the expression of genes through epigenetic regulation (40, 41). Hence, we validated that HULC was capable of inducing the methylation of CpG islands in the promoter of miR-9 (Fig. 4 and Supplementary Fig. S41). This finding suggests that HULC governs the expression of PPARA through epigenetic regulation. The methylation of miR-9-3 is significantly associated with an increased risk of recurrence, and high methylation levels of either miR-9-1 or miR-9-3 result in a significant decrease in recurrence-free survival times in clear cell renal cell carcinoma (36). Moreover, the inhibition of miR-9-mediated suppression of SOX2 is involved in chemoresistance and cancer stemness in glioma cells (42), and miR-9 is able to target MTHFD2 to inhibit the proliferation of breast cancer cells (43). Together with these findings, our data imply that the methylation and inhibition of miR-9 might hijack other signaling pathways to facilitate hepatocarcinogenesis. To better understand the underlying mechanism by which HULC elicits the methylation of CpG islands in the miR-9 promoter, we assessed the influence of HULC on methylase levels in hepatoma cells. Strikingly, our observations indicated that HULC was able to upregulate the expression of DNMT1 in hepatoma cells, suggesting that HULC induces the methylation of miR-9 promoter CpG islands possibly by upregulating DNMT1. The role of ACSL1 in the growth of hepatoma cells has not been reported. Therefore, we examined the effect of ACSL1 overexpression on the proliferation of HepG2 and HuH7 cells by MTT assays and cloning formation assays. Notably, we observed that ACSL1 was able to promote the proliferation of hepatoma cells, suggesting that the role of ACSL1 in the promotion of cell proliferation might be associated with the disturbance of lipid metabolism mediated by ACSL1 in hepatoma cells. In addition, it has been reported that the AKT/mTOR cascade influences the growth, survival, metabolism, and migration of liver cancer cells, and the extent of aberrant lipogenesis is correlated with the activation of the AKT/mTOR signaling pathway (14, 44). This relationship suggests that the AKT/mTOR pathway plays a pivotal role in the deregulation of lipid metabolism in HCC. Therefore, we wondered whether the AKT/mTOR pathway and other signaling pathway members implicated in lipid metabolism, such as SREBP1/2, chREBP, HMGCK, MVK, FASN, ACLY, SQS, and miR-122 (14, 36), were involved in this event. However, we failed to demonstrate that HULC disturbed lipid metabolism in hepatoma cells through these signaling pathways. Therefore, we conclude that HULC is able to induce aberrant lipid metabolism through ACSL1/miR-9/PPARA/ASCL1 signaling in hepatoma cells.

Given that cancer disrupts cellular homeostasis and creates many new methods of regulation, such as positive feedback loops (45), we are interested in whether the action of HULC is involved in a feedback loop as well. Interestingly, we observed that ACSL1 might be responsible for the activation of HULC. Indeed, luciferase reporter assays revealed that cholesterol was able to activate the promoter of HULC, whereas triglycerides were not. Furthermore, we identified a binding site for RXRA in the promoter of HULC using bioinformatics analysis. RXRA is an orphan receptor that can be activated by sterol (22). Thus, we speculated that RXRA might participate in cholesterol-stimulated HULC in hepatoma cells. As expected, we found that RXRA was responsible for the activation of HULC mediated by cholesterol in hepatoma cells. It has been reported that PPARA, liver X receptor (LXRA), and bile acid receptor (FXR) can form heterodimers with RXRA (46–48). Here, we treated cells with siRNA targeting PPARA (or LXRA and FXR) mRNA to assess the effect of PPARA (or LXRA and FXR) on HULC. However, our data demonstrated that all treatments failed to influence the cholesterol-enhanced luciferase reporter activity of the HULC promoter (data not shown). Implying that PPARA, LXRA, and FXR are not implicated in cholesterol-induced RXRA activation. Strikingly, we observed that cholesterol failed to upregulate HULC in normal liver cells (Fig. 5 and Supplementary Fig. S5). The reason may be related to RXRA not being constitutively phosphorylated, thus it fails to escape from Ub/proteasome-mediated degradation in liver cells (49). Of particular note, this finding provides new insights into the mechanism by which HULC is highly expressed in hepatoma cells. Recent reports pinpoint that the growth-promoting effects of elevated levels of insulin, glucose, or triglycerides are involved in
insulin resistance–promoted colorectal cancer (50). Heightened intracellular levels of triglyceride and their metabolites, such as diacylglycerol, may activate the protein kinase-C and MAPK pathways with potentially mitogenic and carcinogenic effects (51). Consistent with our study, these findings suggest that HULC–enhanced accumulation of triglycerides in hepatoma cells might emerge as a risk factor for hepatocarcinogenesis. In this article, we found that cholesterol was involved in the promotion of hepatoma cell growth mediated by HULC, which is consistent with other reports (25–27).

In aggregate, our work demonstrates that HULC plays pivotal roles in aberrant lipid metabolism in HCC through miR-9/PPARA/ACSL1/cholesterol/RXRA/HULC signaling, a model for this role of HULC is represented in Supplementary Fig. S7F. In particular, our data show that HULC elicits the methylation of CpG islands in the miR-9 promoter, resulting in the suppression of miR-9 expression. MiR-9 is able to target the 3’UTR of transcription factor PPARA, and the decrease in miR-9 leads to upregulation of PPARA and the subsequent transactivation of ACSL1, which enhances lipogenesis and enriches intracellular triglycerides and cholesterol in hepatoma cells. Thus, HULC–enhanced abnormal lipid metabolism accelerates the growth of liver cancer. Furthermore, the cholesterol product of ACSL1 upregulates HULC by activating the transcription factor RXRA, forming a positive feedback loop with HULC/miR-9/PPARA/ACSL1/cholesterol/RXRA/HULC in hepatoma cells. Thus, our findings provide new insights into the mechanism of abnormal lipid metabolism mediated by HULC in the development of HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Cui, L. Ye, X. Zhang

Development of methodology: M. Cui, X. Zhang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Cui, Z. Xiao, Y. Wang, M. Zheng, T. Song, X. Cai, B. Sun, L. Ye, X. Zhang

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References


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