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Involvement of cholesterol in hepatitis B virus X protein-induced abnormal lipid metabolism of hepatoma cells *via* up-regulating miR-205-targeted ACSL4

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ABSTRACT

Hepatitis B virus X protein (HBx) plays crucial roles in the development of hepatocellular carcinoma (HCC). The abnormal lipid metabolism is involved in the hepatocarcinogenesis. We previously reported that HBx suppressed miR-205 in hepatoma cells. In this study, we supposed that HBx-decreased miR-205 might contribute to the abnormal lipid metabolism according to the bioinformatics analysis. Interestingly, we showed that the expression levels of acyl-CoA synthetase long-chain family member 4 (ACSL4) were negatively associated with those of miR-205 in clinical HCC tissues. Then, we validated that miR-205 was able to inhibit the expression of ACSL4 at the levels of mRNA and protein through targeting its 3′UTR. Strikingly, we found that HBx was able to increase the levels of cellular cholesterol, a metabolite of ACSL4, in hepatoma cells, which could be blocked by miR-205 (or Triacsin C, an inhibitor of ACSL4). However, anti-miR-205 could increase the levels of cholesterol in the cells. Moreover, we demonstrated that the levels of cholesterol were increased in the liver of HBx transgenic mice in a time course manner. Functionally, oil red O staining revealed that HBx promoted lipogenesis in HepG2 cells, which could be abolished by miR-205 (or Triacsin C). However, anti-miR-205 was able to accelerate lipogenesis in the cells. Interestingly, the treatment with Triacsin C could remarkably block the role of anti-miR-205 in the event. Thus, we conclude that miR-205 is able to target ACSL4 mRNA. The HBx-depressed miR-205 is responsible for the abnormal lipid metabolism through accumulating cholesterol in hepatoma cells.

1. Introduction

The infection of hepatitis B virus (HBV) is closely associated with the development of hepatocellular carcinoma (HCC), in which hepatitis B virus X protein (HBx) plays crucial roles in hepatocarcinogenesis [1–3]. It has been reported that the abnormal lipid metabolism contributes to the event. HBx is able to accelerate the lipogenesis through up-regulating oncogene Rab18 [4]. However, the underlying mechanism by which HBx results in the aberrant lipid metabolism remains poorly understood. As a kind of sterol, cholesterol leaps out from the insoluble members, as it is required by mammals for the synthesis of steroid hormones and bile acids, for the organization of cell membranes, and for the formation and maintenance of lipid rafts [5,6]. More specifically, secreted apoA-I binding protein (AIBP) positively regulates cholesterol efflux from endothelial cells and the effective cholesterol efflux is critical for proper angiogenesis [7]. Recently, it has been reported that the primary metabolite of cholesterol, 27-hydroxycholesterol (27HC), contributes to the estrogen receptor-dependent growth and liver X receptor-dependent metastasis in mouse models of breast cancer [8]. The development of liver cancer might be associated with the accumulation of cholesterol in the tissues as well [9]. However, the mechanism of cholesterol enrichment in hepatoma cells is not well documented. MiR-205 is a tumor suppressor in multiple cancers, such as breast cancer and prostate cancer [10,11]. We previously reported that HBx was able to suppress the expression of miR-205 in hepatoma cells

Abbreviations: HBx, hepatitis B virus X protein; miRNA, microRNA; ACSL4, acyl-CoA synthetase long-chain family member 4; HCC, hepatocellular carcinoma; 3'UTR, 3'-untranslated region; qRT-PCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

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[12]. Therefore, we are interested in whether the HBx-depressed miR-205 plays a role in abnormal lipid metabolism in the development of HCC.

In this study, we aim to gain insights into the molecular mechanisms of aberrant lipid metabolism mediated by HBx. Our data show that HBx-depressed miR-205 is responsible for the abnormal lipid metabolism through accumulating cholesterol in hepatoma cells. This finding provides new insights into the mechanism by which HBx leads to abnormal lipid metabolism in the development of liver cancer.

2. Materials and methods

2.1. Patient samples

Twenty-five clinical HCC tissues and their corresponding nearby nontumorous liver tissue ($n = 17$) were attained from Tianjin First Center Hospital (Tianjin, China). Written consents approving the use of their tissues for research purposes were obtained from the patients. The study protocol was approved by the Institute Research Ethics Committee at the Nankai University.

2.2. Cell lines and cell culture

Human hepatoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (Gibco, CA, USA). The cells were supplemented with heat inactivated 10% fetal bovine serum (FBS, Gibco, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin in 5% CO_2 at 37 °C.

2.3. Plasmid construction

A 200 bp fragment of ACSL4 3′UTR was subcloned into pGL3-control vector (Promega Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene to generate pGL3-ACSL4. Mutant construct of ACSL4 3′UTR (named as pGL3-ACSL4-mut), containing a substitution of 9 nucleotides in the core seed sequence of miR-205, was conducted by using overlapping extension PCR. The primers used in this study for construction were as follows: pGL3-ACSL4 forward, 5′-GCTCTAGAGTTGCTT ATGTTGTTTTGT AC-3, reverse, 5′-GGGGGCCGGCCATCAGTTGGCAG TGTTCTA-3′; pGL3-ACSL4-mut forward, 5′-AAGTATTATAAACAAC TTACTTCCTTTTTGTAAAGATTT-3, reverse, 5′-AAATCTTTACAAAAAG GAAGTAAGT TGTTTAT AATACTT-3′.

2.4. Cell transfection

The cell lines were cultured in a 6-well or 24-well plate for 24 h and then were transfected with 1 μg plasmids (or 50, 100 nM miRNA). All transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. MiR-205 (or anti-miR-205), and miRNA control (miRNA Ctrl) were synthesized by RiboBio (Guangzhou, China).

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR), reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the cells (or tissues) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For mature miR-205 detection, total RNA was polyadenylated by poly (A) polymerase (Ambion, Austin, TX, USA) as described previously [13]. Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The qRT-PCR was performed as described in the method of Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH Mannheim, Germany). The primers used were as follows: ACSL4 forward, 5′-ATTCTTCTCCGCTTACACTC-3′, reverse, 5′-CCTTCTTGCCAGTCTTTT AG-3′; GAPDH forward, 5′-CATCACCATCTTCCAGGAGCG-3′, reverse, 5′-TGACCTTGCCCAC AGCCTTG-3′; miR-205 forward, 5′-TCCTTCATT CCACCGGAGTCTG-3′, reverse, 5′-GCGAGCACAGAATTAATACGA C-3′; U6 forward, 5′-AGAGCCTGTGGTGTCCG-3′, reverse, 5′-CATCTT CAAAGCACTTCCCT-3′.

2.6. Luciferase reporter gene assays

Luciferase reporter gene assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were maintained in 24-well plates at about 3×10^4 cells per well. After 24 h, the cells were transiently co-transfected with the pRL-TK plasmid (Promega, Madison, WI, USA) containing the Renilla luciferase gene, which is used for internal normalization, and with various constructs containing the seed sequence or mutant seed sequence of ACSL4 3′UTR, or pGL3-control. All experiments were performed at least three times.

2.7. Western blotting

The western blotting protocol was described previously [2]. The primary antibodies used were rabbit anti-ACSL4 (Proteintech Group, USA) and mouse anti-β-actin (Sigma, St. Louis, MO, USA). All experiments were repeated three times.

2.8. Oil red O staining

Cells were seeded in 6-well plates and incubated overnight. Cells treated with plasmids or Triacsin C for 48 h were washed twice with phosphate saline and fixed with 10% formalin. The oil red O staining was performed according to the manufacturer's instructions.

2.9. Total cholesterol assay

The levels of cholesterol in cellular and tumorous (obtained from the transgenic mouse) were assayed using Tissue total cholesterol assay kit (Applygen Technologies Inc., Beijing, China). All experiments were performed according to the manufacturer's recommended protocol.

2.10. Statistical analysis

Each experiment was repeated at least three times. Statistical significance was assessed by comparing mean values (6 standard deviation; SD), using a Student's t test for independent groups, and was assumed for $^{**}P < 0.01$, No significant (NS). Pearson's correlation coefficient was used to determine the correlation between the expressions of each gene in tumorous tissues.

3. Result

3.1. The expression levels of miR-205 are negatively correlated with those of ACSL4 in clinical HCC samples

Previously, our laboratory reported that HBx down-regulated miR-205 in hepatoma cells [12]. In this study, we are interested in whether miR-205 plays a role in abnormal lipid metabolism. Therefore, we further screened target genes of miR-205 using Targetscan and microRNA.org (http://www.targetscan.org/, http://

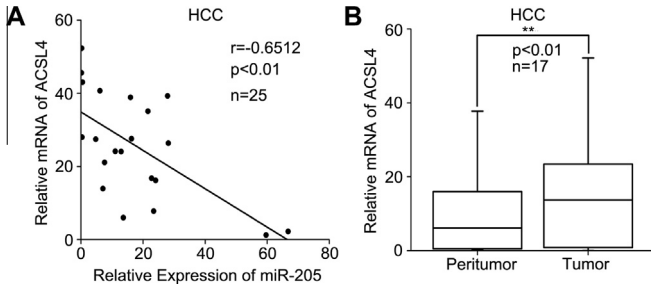

Fig. 1. The expression levels of miR-205 are negatively correlated with those of ACSL4 in clinical HCC samples. (A) Correlation of miR-205 levels with ACSL4 mRNA levels was examined by qRT-PCR in 25 cases of HCC clinical tissues (**$P < 0.01$; Pearson's correlation coefficient, $r = -0.6512$). (B) Relative mRNA levels of ACSL4 were examined by qRT-PCR in 17 pairs of HCC clinical tissues and corresponding nontumorous tissues (**$P < 0.01$; Wilcoxon's signed-rank test).

www.microrna.org). Interestingly, we observed that ACSL4 was a predicted target gene of miR-205. Due to ACSL4 is involved in the steroid biosynthesis in liver tissue [14,15], we concern whether ACSL4 participates in HBx-induced abnormal lipid metabolism. Then, we evaluated the correlation of miR-205 expression with that of ACSL4 in clinical HCC tissues. As expected, our data showed that the expression levels of miR-205 were negatively associated with those of ACSL4 in the tissues (Fig. 1A). Moreover, real-time PCR indicated that the mRNA levels of ACSL4 were higher in HCC tissues comparing with their adjacent nontumorous liver tissues (Fig. 1B). Taken together, our data illustrate that the expression levels of miR-205 anticorrelate with those of ACSL4 in clinical HCC samples.

3.2. MiR-205 is capable of down-regulating ACSL4 via targeting its 3′UTR in hepatoma cells

According to the bioinformatics analysis above, we try to validate whether miR-205 is able to down-regulate ACSL4 in hepatoma cells. As expected, miR-205 was able to down-regulate ACSL4 at the levels of mRNA and protein in a dose-dependent manner (Fig. 2A). In addition, the inverse outcome was exhibited when the cells were treated with anti-miR-205 (Fig. 2B), suggesting that miR-205 can down-regulate the expression of ACSL4 in hepatoma cells. Meanwhile, the transfection efficiency of miR-205 (or anti-miR-205) was validated by qRT-PCR in the cells (data not shown). Next, we aimed to explore the mechanism by which miR-205 down-regulates ACSL4. In Fig. 2C, bioinformatics analysis showed that there was a miR-205-binding site in the 3′UTR of ACSL4. Next, the predicted ACSL4 target site (or mutant target site) sequence by miR-205 in the 3′UTR of ACSL4 was cloned into the downstream of pGL3-control luciferase reporter gene vector (termed pGL3-ACSL4 or pGL3-ACSL4-mut), respectively (Fig. 2D). After the cotransfection of miR-205 with pGL3-ACSL4 was performed in HepG2 cells, the luciferase activity of pGL3-ACSL4 was significantly decreased

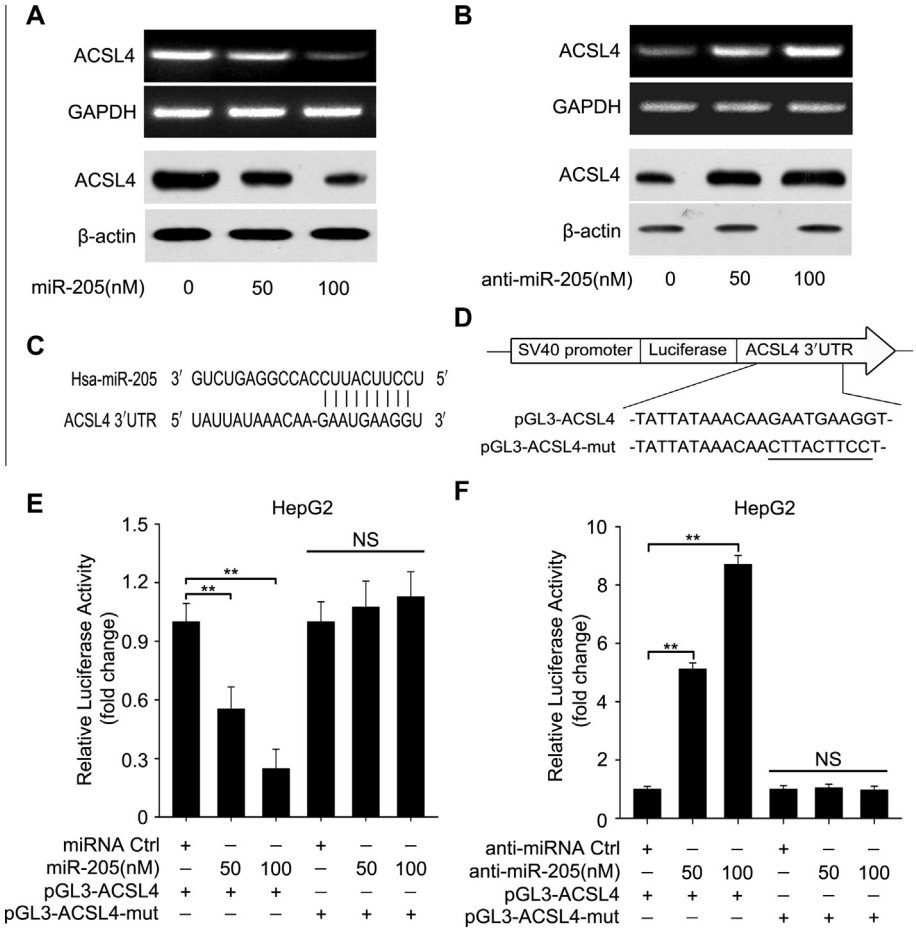

Fig. 2. MiR-205 is capable of down-regulating ACSL4 via targeting its 3′UTR in hepatoma cells. (A) The mRNA and protein levels of ACSL4 were assessed in HepG2 cells transfected with miR-205 by RT-PCR and Western blotting, respectively. (B) The mRNA and protein levels of ACSL4 were examined in HepG2 cells transfected with anti-miR-205 by RT-PCR and Western blotting, respectively. (C and D) A model shows the predictive binding site of miR-205 in ACSL4 mRNA 3′UTR. Schematic diagram displays the generated mutant site at the ACSL4 3′UTR seed region binding to miR-205 and the inserted sites of wild type ACSL4 3′UTR (or mutant) into the downstream of the luciferase reporter gene in pGL3-control vector. (E and F) The effect of miR-205 (or anti-miR-205) on reporters of pGL3-ACSL4 and pGL3-ACSL4-mut in HepG2 cells was measured by luciferase reporter gene assays. Statistically significant differences are indicated: **$P < 0.01$; NS; Student's t test. All experiments were repeated at least three times.

in a dose-dependent manner, but the pGL3-ACSL4-mut failed to work (Fig. 2E). In addition, the inhibition of endogenous miR-205 using anti-miR-205 increased the firefly luciferase activity of pGL3-ACSL4 reporter, but could not influence the activity of pGL3-ACSL4-mut in the cells (Fig. 2F). Therefore, we conclude that miR-205 is able to down-regulate ACSL4 through targeting its 3′UTR in hepatoma cells.

3.3. HBx enriches cholesterol in hepatoma cells and HBx-Tg mice through suppressing miR-205 targeting ACSL4

ACSL4 is an essential enzyme in the steroid synthesis [16]. HBx down-regulates miR-205 in hepatoma cells [12]. Accordingly, we examined the effect of HBx on the levels of cholesterol, a kind of sterol, in hepatoma cells. Strikingly, we observed that HBx was able to accumulate cholesterol in HepG2 cells, which could be restrained by miR-205 or Triacsin C (an inhibitor of ACSL4) in the cells (Fig. 3A). Meanwhile, Triacsin C was capable of eliminating the enrichment of cellular cholesterol induced by anti-miR-205 (Fig. 3B), suggesting that HBx-depressed miR-205 leads to the aberrant metabolism of cholesterol *via* modulating ACSL4 *in vitro*. Interestingly, we demonstrated that the levels of cholesterol were significantly elevated in the liver tissues from HBx transgenic mice (HBx-Tg mice, each sample from 3 mice) relative to those in wild-type (WT) mice littermates (Fig. 3C). Meanwhile, the up-regulation of ACSL4 was validated in HBx-Tg mice relative to WT mice (Fig. 3C), which were consistent with the levels of cholesterol in the system. Thus, we conclude that HBx accumulates cholesterol in hepatoma cells and liver tissues of HBx-Tg mice through suppressing miR-205 targeting ACSL4.

3.4. HBx mediates dysregulation of lipogenesis through miR-205 targeting ACSL4

Lipid droplets regulate the storage and hydrolysis of neutral lipids, such as cholesterol ester [17]. The oil red O staining revealed

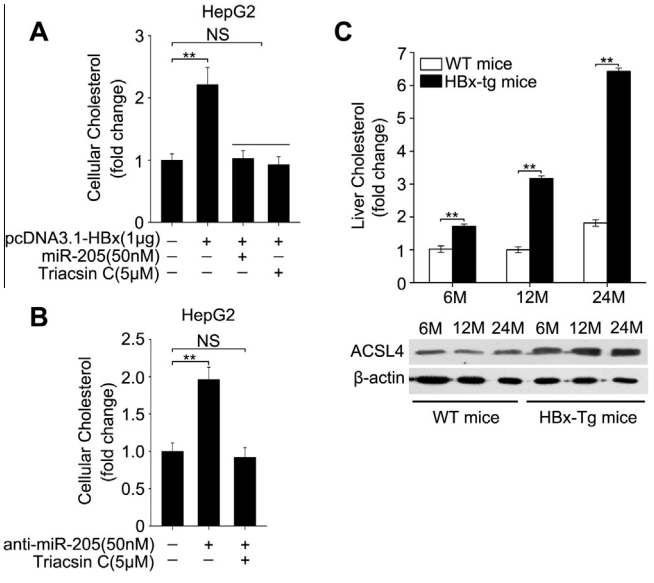

Fig. 3. HBx enriches cholesterol in hepatoma cells and HBx-Tg mice through suppressing miR-205 targeting ACSL4. (A) Effect of HBx (or HBx and miR-205, HBx and Triacsin C) on cellular cholesterol was measured in HepG2 cells by Tissue total cholesterol assay kit. (B) Effect of anti-miR-205 (or anti-miR-205 and Triacsin C) on cellular cholesterol was measured in HepG2 cells as above. (C) Levels of cholesterol were measured by Tissue total cholesterol assay kit in the liver tissues from HBx-Tg mice and WT littermates aged 6, 12 and 24 month (M). The expression levels of ACSL4 in the system were assessed by Western blotting. Statistically significant differences are indicated: $^{**}P < 0.01$, NS; Student's *t* test.

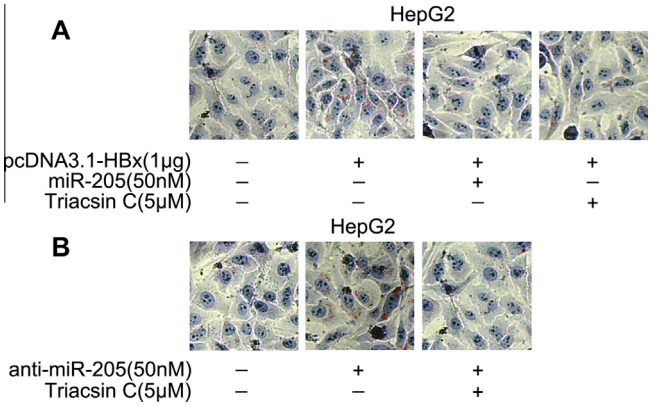

Fig. 4. HBx mediates dysregulation of lipogenesis through miR-205 targeting ACSL4. (A) Effect of HBx (or HBx and miR-205, HBx and Triacsin C) on lipogenesis was determined by oil red O staining in HepG2 cells. (B) Effect of anti-miR-205 (or anti-miR-205 and Triacsin C) on lipogenesis was determined by oil red O staining in HepG2 cells.

that HBx promoted lipogenesis in HepG2 cells, which could be abolished by miR-205 (or Triacsin C) (Fig. 4A). However, anti-miR-205 was able to accelerate lipogenesis in the cells. Interestingly, the treatment with Triacsin C could remarkably block the role of anti-miR-205 in the event (Fig. 4B). It suggests that HBx induces abnormal lipid metabolism in hepatoma cells through miR-205 targeting ACSL4.

4. Discussion

Vast studies have pointed out that HBx hijacks host genes expression and contributes to apoptosis, epigenetic, metastasis and inflammation [18–21]. Recently, Shin has corroborated that HBx takes part in regulating the homeostasis of hepatic glucose [22]. Our group has indicated that HBx accelerates lipogenesis through up-regulating Rab18 in HCC [4], suggesting that HBx contributes to the metabolism reprogramming in tumorigenesis. However, the underlying mechanism by which HBx modulates aberrant lipid metabolism remains ill-identified.

Due to abnormal lipid metabolism acts major roles in HCC [23], in this study we aim to explore how HBx participates in the event. Previous studies demonstrated that adipokine [24], insulin resistance [25], virus infection [26] and autophagy [27] were involved in the aberrant lipid metabolism. In addition, mounting researches have focused on the effect of miRNAs on abnormal lipid metabolism [28,29]. Our group has reported that HBx down-regulates tumor suppressor miR-205 to promote HCC [12]. Hence, we are obsessed with whether HBx governs the deregulation of lipid metabolism through inhibiting miR-205 in the liver cancer. We scrutinized the target genes of miR-205 using bioinformatics tools. Strikingly, ACSL4 was prominent since its function and expression pattern. As expected, our observation revealed that low levels of miR-205 negatively correlated with high levels of ACSL4 in HCC clinical samples (Fig. 1). Moreover, we identified that miR-205 was capable of down-regulating ACSL4 in hepatoma cells through directly binding to its 3′UTR (Fig. 2).

ACSL4 acts an essential role in steroid synthesis [16]. As a sterol, cholesterol attracts a mass of attentions since its bioactivity. Given that cholesterol appears to be required in the cell proliferation [30]. High circulating cholesterol increases risk of aggressive prostate cancer [31]. On the basis of the conclusions, we were interested in whether HBx activates the biosynthesis of cholesterol through miR-205 targeting ACSL4. Strikingly, our data demonstrated that miR-205 or Triancsin C (an inhibitor of ACSL4) was

able to prevent the accumulation of cellular cholesterol mediated by HBx or anti-miR-205. In addition, the HBx-Tg mice were used to validate the outcomes. Inevitably, the levels of cholesterol in liver tissues were consistent with those of ACSL4 in the mouse models (Fig. 3). It suggests that HBx raises the levels of cholesterol *via* miR-205 targeting ACSL4. Next, we concerned whether HBx participated in the deregulation of lipid metabolism through miR-205 targeting ACSL4. Oil red O staining showed that miR-205 could eliminate HBx-induced lipogenesis. Moreover, Triacsin C was able to block the increase of lipogenesis induced by HBx or anti-miR-205 (Fig. 4). It suggests that HBx contributes to the abnormal lipid metabolism through down-regulating miR-205 targeting ACSL4. Previous report demonstrated that ACSL4 was overexpressed in breast, colon, prostate and liver cancer [32]. In this study, we conclude that HBx is capable of deregulating lipogenesis, especially cholesterol metabolism, through ACSL4 in the development of HCC.

In summary, our finding first illustrates that HBx enriches cholesterol in hepatoma cells. HBx-decreased miR-205 results in overexpression of ACSL4, leading to the acceleration of lipogenesis. Thus, our finding provides new insights into the mechanism of HBx in the abnormal lipid metabolism of liver cancer.

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