

# Role of ATP-binding cassette transporter A1 in suppressing lipid accumulation by glucagon-like peptide-1 agonist in hepatocytes



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## ABSTRACT

**Objective:** Adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) influences hepatic cholesterol transportation. Accumulation of hepatic cholesterol leads to fatty liver disease, which is improved by glucagon-like peptide 1 (GLP-1) in diabetes. Therefore, we analyzed the molecular mechanism in the regulation of hepatic ABCA1 by GLP-1 analogue exendin-4.

**Methods:** Hepatic ABCA1 expression and transcription were checked by western blotting, real-time polymerase chain reaction (PCR), and luciferase assay in HepG2 cells. Chromatin immunoprecipitation (ChIP) and site-directed mutagenesis were employed to determine transcriptional regulation of the ABCA1 gene. Prolactin regulatory element-binding (PREB)-transgenic mice were generated to access the effect of exendin-4 on improving lipid accumulation caused by a high-fat diet (HFD).

**Results:** Exendin-4 stimulated hepatic ABCA1 expression and transcription via the Ca<sup>2+</sup>/calmodulin (CaM)-dependent protein kinase kinase/CaM-dependent protein kinase IV (CaMKK/CaMKIV) pathway, whereas GLP-1 receptor antagonist exendin9-39 cancelled this effect. Therefore, exendin-4 decreased hepatic lipid content. ChIP showed that PREB could directly bind to the ABCA1 promoter, which was enhanced by exendin-4. Moreover, PREB stimulated ABCA1 promoter activity, and mutation of PREB-binding site in ABCA1 promoter cancelled exendin-4-enhanced ABCA1 promoter activity. Silencing of PREB attenuated the effect of exendin-4 and induced hepatic cholesterol accumulation. Blockade of CaMKK by STO-609 or siRNA cancelled the upregulation of ABCA1 and PREB induced by exendin-4. *In vivo*, exendin-4 or over-expression of PREB increased hepatic ABCA1 expression and decreased hepatic lipid accumulation and high plasma cholesterol caused by a HFD.

**Conclusions:** Our data shows that exendin-4 stimulates hepatic ABCA1 expression and decreases lipid accumulation by the CaMKK/CaMKIV/PREB pathway, suggesting that ABCA1 and PREB might be the therapeutic targets in fatty liver disease.

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**Keywords** Prolactin regulatory element-binding; Lipid accumulation; Non-alcoholic steatohepatitis; Fatty liver; Cell signaling

## 1. INTRODUCTION

The liver is one of the important organs related to diverse metabolic activities, including lipid metabolism. Equipped with varying enzymes, hepatocytes function efficiently in many lipid processes, such as lipid uptake, lipid *de novo* synthesis and oxidation, and lipid export. As part of this metabolic flux, excess intracellular lipids are primarily stored in triglyceride (TG)-enriched lipid droplets in the cytoplasm. Abnormal accumulation of hepatic lipid droplets occurs in numerous pathologic conditions, such as alcoholic liver disease, hepatitis C, and non-alcoholic fatty liver disease (NAFLD) [1]. NAFLD is always associated with hepatic

steatosis, which is defined as the abnormal accumulation of lipids in the liver, especially TG, contributing over 5% of the liver weight [2]. A balanced steady state of lipid in the liver contributes to the pathogenesis of hepatic steatosis, including lipogenesis, lipolysis, and fatty acid oxidation [3]. Recent reports suggest that by 2020, NAFLD will become the leading cause of liver transplantation [4,5]. Newer agents are shown to improve liver histology in NAFLD, and glucagon-like peptide-1 receptor (GLP-1R) agonists have recently exhibited an attractive therapeutic option for patients with diabetes and NAFLD [6]. Clinically, insulin secretagogue hormone GLP-1 and GLP-1R long-acting agonist exendin-4 have been demonstrated to stimulate

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**Abbreviations:** ABCA1, ATP-binding cassette transporter A1; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HDL, high density lipoprotein; ChIP, Chromatin immunoprecipitation; PREB, prolactin regulatory element-binding protein; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PKC, protein kinase C; CaMKK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase; ERK, extracellular signal-regulated kinase; HDL-C, HDL cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; CREB, cAMP-responsive element binding; HCV, hepatitis C virus

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glucose-dependent insulin secretion and lower the blood glucose levels in people with type 2 diabetes [7]. Metabolic syndromes such as type 2 diabetes and obesity are well known to be closely associated with the pathology of the liver. In clinical trials on type 2 diabetes, exendin-4 decreased food intake and body weight, especially in patients with obesity [8]. Recently, high-fat diet (HFD)-fed mice treated with exendin-4 exhibited a decrease of the net weight gained, improved serum glucose, and reduced hepatic steatosis with well-improved insulin sensitivity [9,10]. However, it is not clear how exendin-4 protects the hepatocytes from steatosis.

ATP-binding cassette transporter A1 (ABCA1), a 254-kD membrane protein, is a pivotal regulator of lipid efflux from the cytoplasm to apolipoproteins, playing an important role in reverse cholesterol transport [11,12]. It is identified as a mutated molecule in Tangier disease, and the absence of ABCA1 induces severe high-density lipoprotein (HDL) deficiency, the deposition of cholesterol in tissue, and premature coronary atherosclerosis [13]. ABCA1 is widely expressed in many tissues, such as the pancreas and the liver. Mice with specific inactivation of the ABCA1 gene in the pancreas showed altered cholesterol homeostasis, markedly impaired glucose tolerance, and defective insulin secretion [14,15]. Specific overexpression of ABCA1 in the mouse liver increased plasma HDL concentration and changed hepatic cholesterol efflux [16,17], whereas deletion of liver-specific ABCA1 decreased the concentration of HDL to 17% of the normal level and increased the secretion of TG [13], indicating the important roles of ABCA1 in hepatic cholesterol homeostasis.

Previously, we reported that exendin-4 stimulates the expression of pancreatic ABCA1 through  $Ca^{2+}$ /calmodulin (CaM)-dependent protein kinase IV (CaMKIV) cascade and via the prolactin regulatory element-binding (PREB) transcriptional factor [18,19]. The PREB gene encodes 1.9-kb mRNA, which is translated into a transcription factor that binds to the basal prolactin promoter [20]. PREB is ubiquitously expressed in different human tissues, such as the pituitary gland, the pancreas, the liver, and the adrenal gland. In our previous study, PREB has been proved to act as a transcriptional factor and regulate the transcription of the insulin gene by binding to the glucose response element of the insulin promoter [21].

Although abnormal lipid accumulation might be an important comorbidity in NAFLD, the exact roles of ABCA1 in hepatic lipid accumulation have not yet been clarified. In this study, we investigated the detailed mechanisms of how exendin-4 suppresses lipid accumulation by controlling the expression of the ABCA1 gene in hepatocytes.

## 2. MATERIALS AND METHODS

### 2.1. Cell culture

HepG2 (RIKEN Cell Bank, Japan) was grown in Dulbecco modified Eagle minimal essential medium with low glucose (L-DMEM; Gibco) supplemented with 10% heat-inactivated certified Australian fetal bovine serum (FBS) (Thermo Scientific), 100 U/mL penicillin, and 0.1 mg/mL streptomycin as described previously [22].

Primary hepatocytes were isolated from mice as described previously [23]. Briefly, mice were anesthetized by phenobarbital sodium. The liver was perfused with Hank's Balanced Salt Solution (HBSS, Wako) containing 0.5 mM ethylenediamine tetraacetic acid (EDTA) to remove the blood. Then, HBSS containing collagenase (0.5 mg/mL; Wako) was injected into the liver and incubated at 37 °C for 15 min. Next, the liver was torn to release hepatocytes; digestion was stopped with L-DMEM containing 10% FBS; and the liver suspension was passed through a 75-mm strainer. The cells were collected and washed twice with HBSS containing 1% bovine serum albumin (BSA). The cells were plated at

$2 \times 10^5$  cells/mL and cultured with L-DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

After starvation with L-DMEM with 0.5% heat-inactivated FBS for 6 h, HepG2 cells or primary hepatocytes were treated with exendin-4 at varying concentration or exendin9-39 for 24 h. For the treatment with inhibitor, HepG2 cells or primary hepatocytes were first treated with inhibitor or DMSO for 30 min and then treated with exendin-4 at 10 nM for 24 h.

### 2.2. Animals

All animal procedures followed the Guide for Experimental Animal Research and were approved by the Animal Care Committee of Kagawa University. PREB-transgenic (PREB-Tg) mice were generated as described previously [24]. Six-week-old mice were divided into four groups ( $n = 5$  each): normal food diet (NFD); NFD plus 1 nmol/kg/day exendin-4; high-fat diet (HFD); and HFD plus 1 nmol/kg/day exendin-4 (Lilly) via intraperitoneal injection. The HFD contains 22.02% crude protein (~18% calories), 33.21% crude fat (~60% calories), 26.4% nitrogen-free extract (~22% calories), and 6.31% crude fiber. NFD contains 23.1% crude protein (~20% calories), 5.1% crude fat (~10% calories), 55.3% nitrogen-free extract (~70% calories), 5.8% crude ash, and 2.8% crude fiber. The diet lasted for 8 weeks, and exendin-4 treatment was started from the 5th week. The mice were euthanized after overnight fasting.

### 2.3. Western blot

15–30  $\mu$ g proteins were separated by a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride membranes for immunoblotting as described previously [25]. The membranes were blocked overnight and incubated with 3% BSA and 0.1% Tween 20 in PBS (PBS-T) containing rabbit polyclonal antibody first antibody (Santa Cruz Biotechnology, Inc) against ABCA1 (sc-20794; 1:200), p-CaMKIV (sc-28443-R; 1:200), CaMKIV (sc-9036; 1:200), PREB (abcam, ab42501; 1:4000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; TREVIGEN, 2275-PC-100; 1:5000), or transcription factor IID (TFIID; sc-273; 1:200) overnight, and incubated for 1 h with horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG) secondary antibody (DakoCytomation, P0448; 1:2000) at 4 °C. Then, antigen–antibody complexes were visualized by enhanced chemiluminescence (ECL; GE Healthcare).

### 2.4. Real-time reverse transcriptase-polymerase chain reaction

We synthesized cDNA using reverse-transcribed total RNA from HepG2 cells as described previously [26]. Polymerase chain reaction (PCR) was performed with a final volume of 10  $\mu$ L using CFX96 Touch Real Time PCR Detection Systems (BIO-RAD). The forward and reverse primers were: human ABCA1 5'-TTGAATTCTGGGCAAATG-3' and 5'-TGGGGATGCCTTCAAACAC-3' [27]; human PREB 5'-GTCATTTCCTGCCTCACT-3' and 5'-GTCACATCTGTACCACACA-3' [20]. Each set of PCR reactions included water as a negative control and five dilutions of the standard. Known amounts of DNA were then diluted to make the standards, and human beta-actin was used as the housekeeping standard. The resulting values were analyzed as the relative expression compared with control levels as described previously [28].

### 2.5. Transfection and luciferase reporter gene assay

The luciferase reporter plasmid containing the human ABCA1 promoter region (pABCA1-LUC) was constructed, and PREB response sequence (PRS) mutated plasmid (pABCA1-mt-LUC) was generated by site-directed mutagenesis as described previously [29]. HepG2 was

transfected with pABCA1-LUC or co-transfected with a vector expressing a constitutively active form of CaMKIV or dominant-negative mutant of CaMKIV (71 K to E and 316 FN to DD [30]; CaMKIV-DN) plus pABCA1-LUC. Transfected cells were treated with or without 10 nM of exendin-4 for 24 h before harvest, and luciferase activity was checked as previously described [31].

### 2.6. Cholesterol content assay

To measure cellular cholesterol concentration, we used a colorimetric assay that utilizes reagents widely used for the measurement of cholesterol in conjunction with a random access chemistry analyzer, ARCHITECT c8000 [29].

### 2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed by the ChIP-IT™ kit (Active Motif). Chromatin was immunoprecipitated with 2 µg of either PREB antibody (Abcam, ab42501) or negative control IgG. DNA was analyzed by PCR or real-time PCR to amplify the fragment spanning nucleotides -849 to -732 of the human ABCA1 promoter sequence containing PRS by using primers forward 5'-TACACGACTGCAATTCTCTGGCTG-3' and reverse 5'-TGA-GAGGAAGGAGGCCACAAAAC-3' [29].

### 2.8. Transfection of small interfering RNA (siRNA)

siRNAs were designed to target the following cDNA sequences: scrambled, 5'-CCGTTCTGTACAGGGAGTACT-3'; PREB-siRNA, 5'-AATGGCGTGCACCTTCTCGAG-3' [29]. CaMKIV-siRNA (sc-29902) and its scrambled siRNA (sc-36869) were purchased from Santa Cruz.

Transfection of siRNA was performed using siPORT Lipid (Ambion) as described in a previous study [29].

### 2.9. Statistical analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA) and the Student t test;  $P < 0.05$  was considered significant.

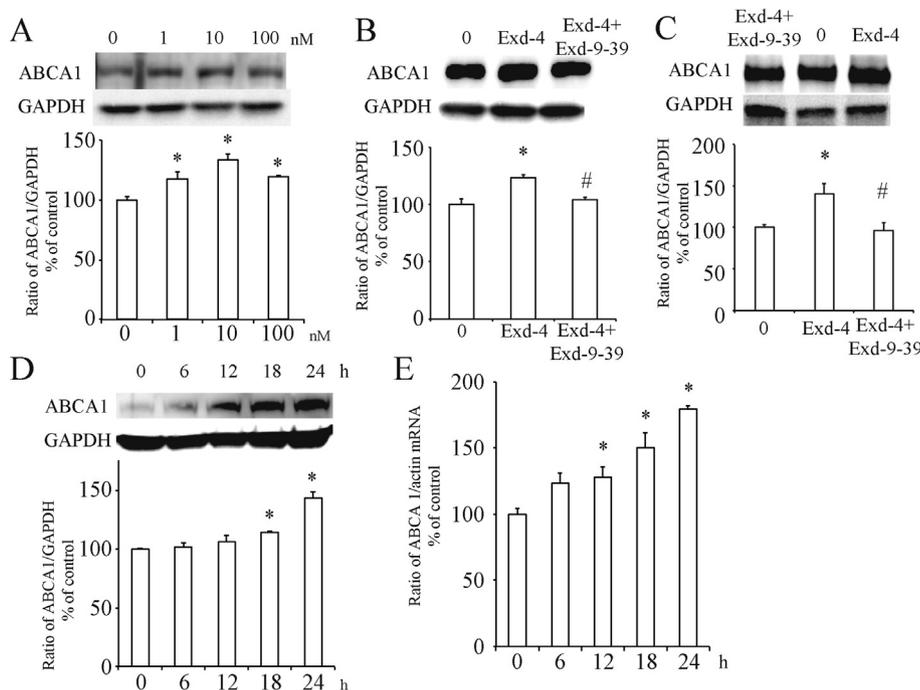
## 3. RESULTS

### 3.1. Exendin-4 increases ABCA1 expression in hepatocytes

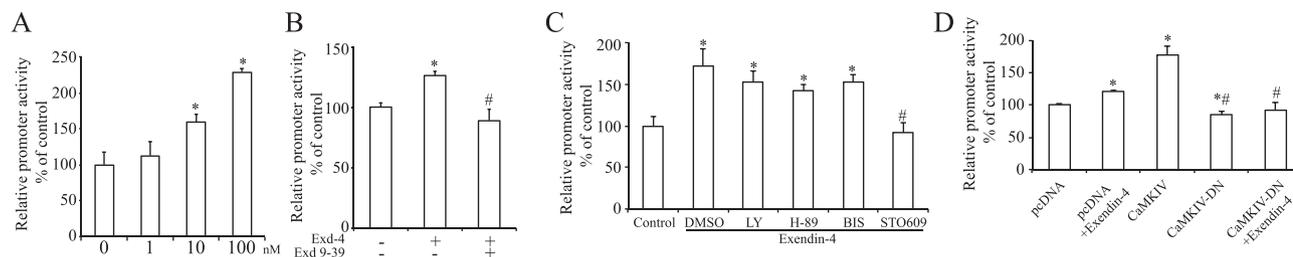
To test the effect of exendin-4 on the expression of ABCA1, HepG2 cells were exposed to exendin-4 at varying concentrations (0–100 nM) for 24 h. Western blotting showed increased abundance of ABCA1 protein in HepG2 cells after treatment with exendin-4 (Figure 1A) without affecting the expression of ABCG1 (Figure S1A). However, the induction of ABCA1 decreased after blocking of GLP-1R by exendin9-39 (Figure 1B). Similar results were obtained in primary hepatocytes isolated from mice (Figure 1C). Next, we treated HepG2 cells with exendin-4 at 10 nM for different times (0–24 h) and found that it time-dependently increased protein and mRNA levels of ABCA1 (Figure 1D,E). These results showed that exendin-4 increased ABCA1 expression via GLP-1R in hepatocytes.

### 3.2. Exendin-4 enhances ABCA1 promoter activity in HepG2 cells via CaMKK/CaMKIV pathway

To check the role of exendin-4 on the transcription of ABCA1, we employed the luciferase report system constructed with ABCA1



**Figure 1:** Effects of exendin-4 on ABCA1 protein expression in HepG2 cells. A, ABCA1 protein expression in HepG2 cells treated with exendin-4 at varying concentrations for 24 h. B, ABCA1 protein expression in HepG2 cells treated with exendin-4 (Exd-4) or exendin-9-39 (Exd 9-39). C, Expression of ABCA1 in primary hepatocytes treated with Exd-4 or Exd 9-39. D, ABCA1 protein expression in HepG2 cells treated with exendin-4 at 10 nM for different times. E, Effect of exendin-4 on ABCA1 mRNA expression. GAPDH/actin served as control, and the ratio of ABCA1 to GAPDH/actin is shown in histogram as percent of control. A histogram shows the mean  $\pm$  SEM ( $n = 3$ ) of separate group. \* $P < 0.05$  compared with 0; # $P < 0.05$  compared with exendin-4.



**Figure 2:** Exendin-4 increases the promoter activity of ABCA1 via CaMKK/CaMKIV signaling pathway. A, ABCA1 promoter activity in HepG2 cells treated with exendin-4 at varying concentrations for 24 h. B, ABCA1 promoter activity in HepG2 cells treated with exendin-4 or exendin9-39. C, Effects of a PI3K inhibitor LY294002 (LY), a PKA inhibitor H-89, a PKC inhibitor bisindolylmaleimide I (BIS), or a CaMKK inhibitor STO-609 (STO) on ABCA1 promoter activity. D, Role of CaMKIV in ABCA1 promoter activity. pcDNA, control; CaMKIV, overexpression of CaMKIV; CaMKIV-DN, domain negative CaMKIV. Each data point shows the mean  $\pm$  SEM (n = 3) of separate group. \*P < 0.05 compared with control; #P < 0.05 compared with exendin-4.

promoter. As shown in Figure 2A, 10 nM and 100 nM of exendin-4 significantly increased the transcriptional activity of ABCA1 promoter, whereas exendin9-39 blocked this effect (Figure 2B).

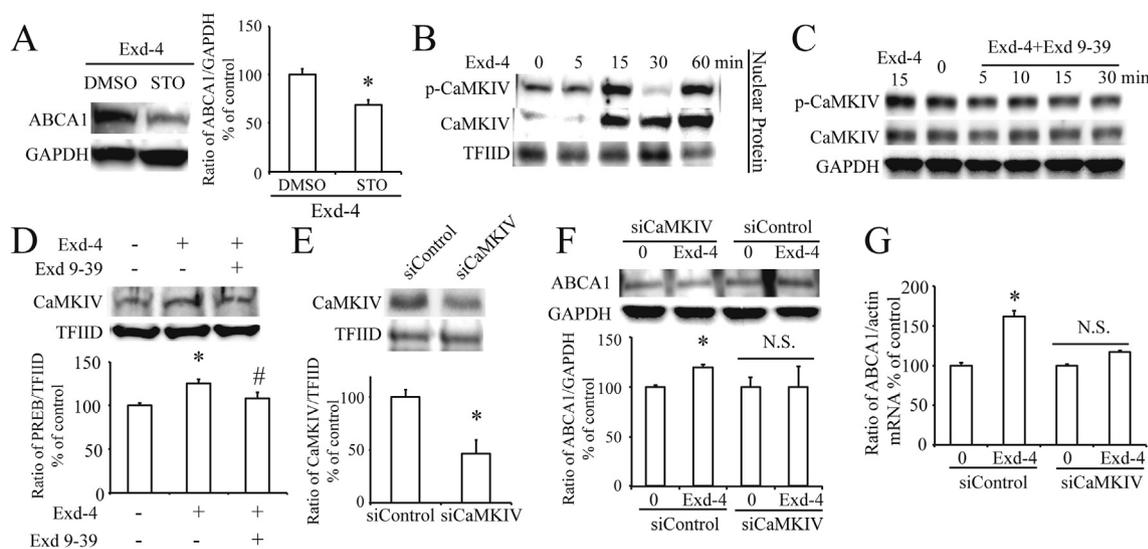
Our previous study showed that by binding to GLP-1R, exendin-4 was able to stimulate multiple signaling pathways including CaMKK [25]. To determine the pathways in exendin-4-enhanced ABCA1 promoter activity, we treated HepG2 cells with inhibitors, which are known to separately block PI3K (10  $\mu$ M LY294002), PKA (1  $\mu$ M H-89), PKC (1  $\mu$ M bisindolylmaleimide I), or CaMKK (1  $\mu$ g/mL STO-609), before exendin-4 treatment. Results showed that inhibitors of PI3K, PKA, or PKC had no effect on the action of exendin-4, but STO-609, the inhibitor of CaMKK, abrogated the ability of exendin-4 to induce ABCA1 promoter activity (Figure 2C).

As a downstream of CaMKK, CaMKIV is demonstrated to regulate gene transcription [32], and the stimulation of CaMKIV by exendin-4 enhances ABCA1 promoter activity in the pancreatic beta cells [18]. Further, we studied the role of CaMKIV in mediating hepatic ABCA1 transcription. As shown in Figure 2D, ABCA1 promoter activity was significantly enhanced by constitutively activating CaMKIV and decreased by domain-negative CaMKIV; and, the addition of exendin-4 could not fully rescue the decrease. These findings support the idea

that the activation of the CaMKK/CaMKIV signaling pathway is required for hepatic ABCA1 transcription induced by exendin-4.

### 3.3. Exendin-4 stimulates ABCA1 expression via CaMKK/CaMKIV signaling pathway

Next, we checked the role of the CaMKK pathway in exendin-4-mediated ABCA1 expression. Consistent with the result of promoter activity, the inhibition of CaMKK with STO-609 decreased exendin-4-induced ABCA1 expression (Figure 3A) without affecting the expression of ABCG1 (Figure S1B). A previous study showed that activated CaMKIV by CaMKK is translocated from the cytoplasm to the nucleus, where it regulates gene expression [32]. Next, we found that CaMKIV was strongly activated at the Thr196 site and recruited to the nucleus by the treatment with exendin-4 from 15 min (Figure 3B). However, activation of CaMKIV was cancelled when GLP-1R was blocked by exendin9-39 (Figure 3C,D). When the nuclear CaMKIV was knocked down by siRNA (Figure 3E), the increased ABCA1 induced by exendin-4 did not persist (Figure 3F,G). These data indicated that exendin-4 could stimulate the CaMKK/CaMKIV signaling pathway, which was involved in the regulation of ABCA1 expression.



**Figure 3:** Activation of CaMKIV induced by exendin-4. A, Effect of STO-609 (STO) on ABCA1 expression induced by exendin-4. B, Nuclear expression of Thr196-phosphorylated CaMKIV or CaMKIV in HepG2 cells treated with exendin-4. C and D, Effect of exendin9-39 on the activation of CaMKIV and its nuclear recruitment induced by exendin-4. E, Silencing of CaMKIV by siRNA. F and G, Effect of exendin-4 on ABCA1 expression after silencing CaMKIV. GAPDH/TFIID/actin served as control. A histogram shows the mean  $\pm$  SEM (n = 3) of separate group. Exd-4, exendin-4 at 10 nM, Exd9-39, exendin9-39 at 10 nM \*P < 0.05 compared with control; #P < 0.05 compared with exendin-4; N.S., not significant.

### 3.4. Exendin-4 decreases lipid accumulation in HepG2 cells via CaMKK pathway

ABCA1, a cholesterol transporter, facilitates cellular cholesterol efflux to HDL in the presence of apolipoprotein A-I (ApoA-I). Consistent with the upregulation of ABCA1, exendin-4 significantly decreased cholesterol content to 90% of control, whereas blocking of GLP-1R by exendin9-39 cancelled this effect (Figure 4A). Next, we found that only the CaMKK inhibitor STO-609 blocked exendin-4-suppressed cholesterol accumulation (Figure 4B) without affecting basal cholesterol contents in the absence of exendin-4 (data not shown). This result is confirmed by oil red O stain (Figure 4C and Figure S2), indicating that the CaMKK pathway is involved in exendin-4-suppressed cholesterol accumulation.

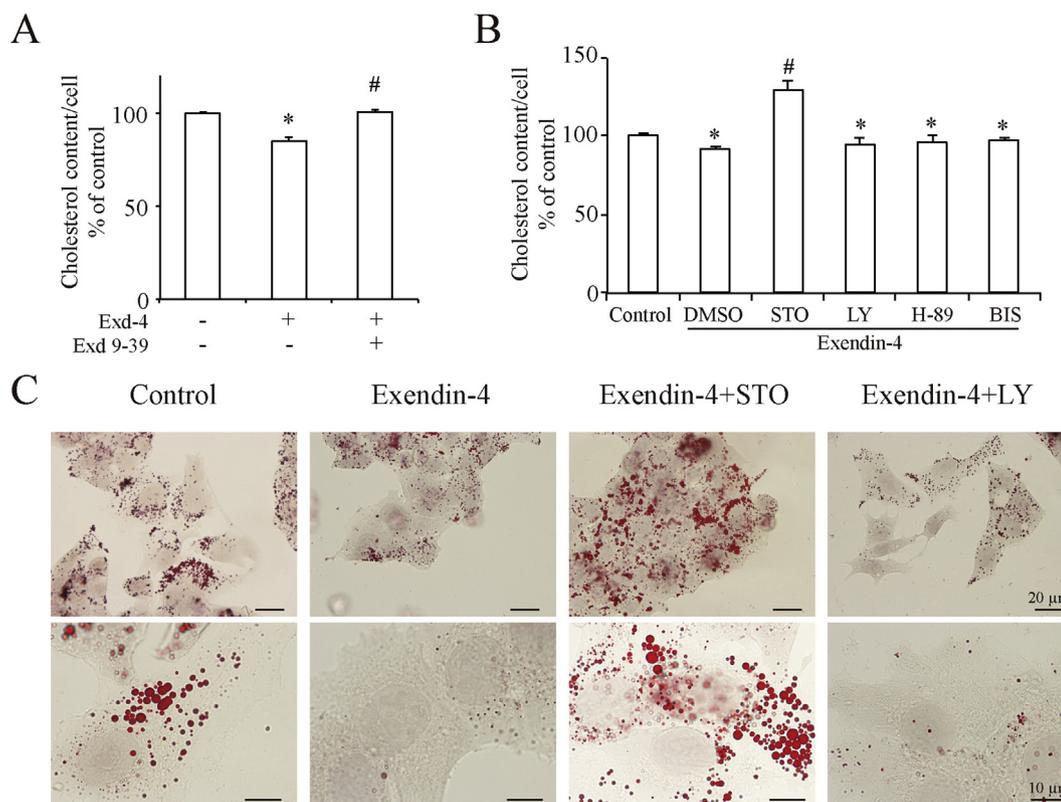
### 3.5. Exendin-4 induces ABCA1 expression and reduces lipid accumulation via PREB in HepG2 cells

Nuclear translocation of CaMKIV is involved in gene transcription through the phosphorylation of certain transcription factors [33]. Our previous study demonstrated that the transcriptional factor PREB regulated the expression of ABCA1 in human aortic smooth muscle cells and rat pancreatic beta cells [19,29]. In this case, we searched for a DNA motif within the ABCA1 promoter that could bind PREB. Examination of the promoter sequence showed a 5-nucleotide motif (CTGAT) corresponding to the deduced PREB core-binding element in the ABCA1 promoter region. Therefore, we employed ChIP assay to demonstrate that PREB could bind to the ABCA1 promoter (Figure 5A). ChIP-real time PCR showed that

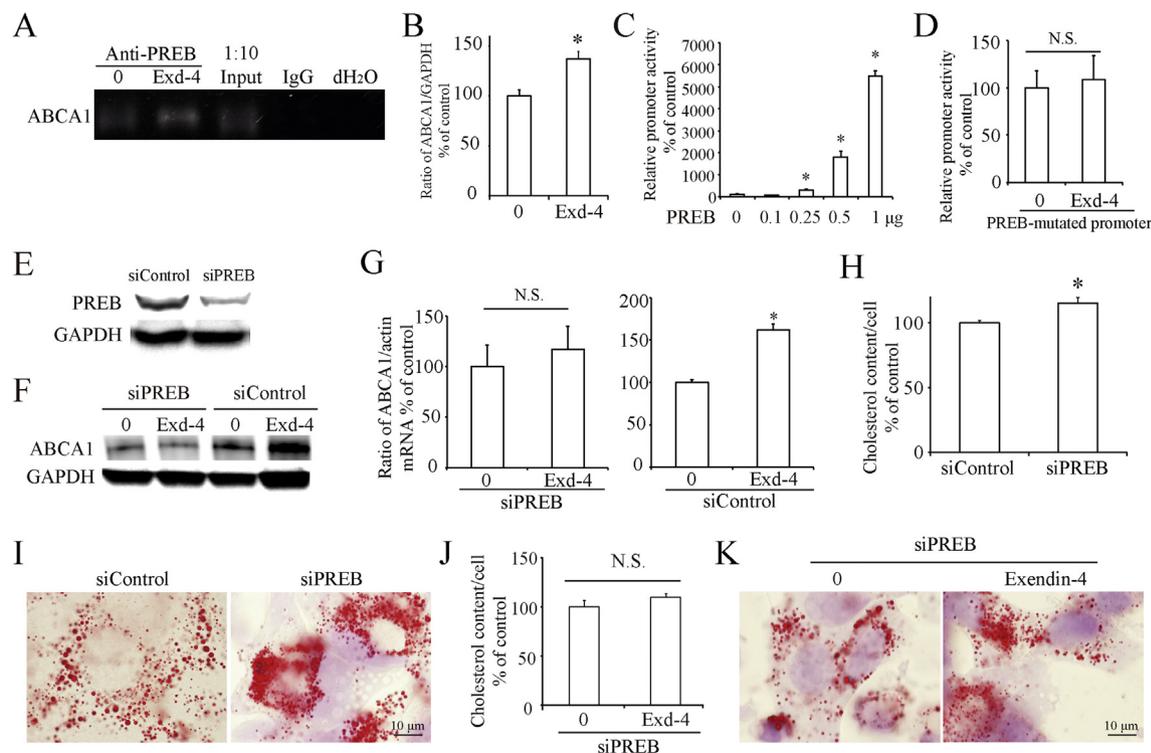
this binding was enhanced by the treatment of exendin-4 (Figure 5B). At the same time, the overexpression of PREB stimulated the ABCA1 promoter activity in a dose-dependent manner (Figure 5C). However, when the PREB-binding site in the ABCA1 promoter was mutated, the stimulatory effect of exendin-4 on ABCA1 promoter activity was abrogated (Figure 5D), suggesting that exendin-4 regulates the transcription of the ABCA1 gene via the transcription factor PREB.

To further characterize the role of PREB in regulating the expression of ABCA1 in HepG2 cells, we used specific siRNA to block PREB expression. As shown in Figure 5E, the expression of PREB was inhibited by PREB-specific siRNA but not by a scrambled siRNA. Next, HepG2 cells exposed to PREB-specific or scramble siRNA were treated with or without exendin-4. We found that silencing PREB cancelled the effects of exendin-4 on the protein and mRNA levels of ABCA1 (Figure 5F,G). Because of ABCA1 reduction, cholesterol and lipid droplets was accumulated in PREB-silenced HepG2 cells compared with the scramble group (Figure 5H,I). Furthermore, the silencing of PREB cancelled the ability of exendin-4 to decrease cholesterol content (Figure 5J,K).

Next, we examined the effect of exendin-4 on the nuclear recruitment of PREB, and found that treatment with exendin-4 promoted the translocation of PREB from the cytoplasm to the nucleus at 5 min and 30 min (Figure 6A). At the same time, exendin-4 significantly increased PREB mRNA level in HepG2 cells (Figure 6B). The results of western blotting also demonstrated that PREB expression was upregulated by exendin-4 in a dose-dependent (Figure 6C) and time-dependent



**Figure 4:** Exendin-4 decreases lipid content in HepG2 cells via CaMKK. Cholesterol content (A and B) or oil red O stain (C) in HepG2 cells with varying treatment. Cholesterol content was normalized by cells number. Exd-4, exendin-4 at 10 nM, Exd9-39, exendin9-39 at 10 nM. LY, PI3K inhibitor LY294002; H-89, PKA inhibitor H-89; BIS, PKC inhibitor bisindolylmaleimide I; STO, a CaMKK inhibitor STO-609. Results are represented as mean  $\pm$  SEM ( $n = 3$ ) of separate group. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with exendin-4.



**Figure 5:** Role of PREB in ABCA1 transcription in HepG2 cells. A and B, Binding of PREB to ABCA1 promoter region. PREB specifically immunoprecipitates the ABCA1 chromatin by ChIP assays and ChIP-real time PCR. IgG, negative IgG. C, Effect of PREB on ABCA1 promoter activity. D, Effect of exendin-4 on PREB binding site-mutated promoter activity (5'-CTGAT-3' to 5'-AGCCT-3'). E, Silencing of PREB with siRNA. F and G, Effect of exendin-4 on protein and mRNA expression of ABCA1 after silencing PREB. H and K, Effects of PREB-knockdown on lipid accumulation in HepG2 cells. siControl, scramble siRNA; siPREB, siRNA specific to PREB. Each data point shows the mean  $\pm$  SEM (n = 3) of separate group. \*P < 0.05; N.S., not significant.

(Figure 6D) manner. However, blocking GLP-1R by exendin9-39 cancelled the effect of exendin-4 on PREB (Figure 6E,F). Furthermore, the inhibition of the CaMKK pathway by STO-609 or silencing of CaMKIV cancelled the induction of PREB by exendin-4 (Figure 6G,H), showing that exendin-4 could stimulate the expression of PREB via the CaMKK/CaMKIV signaling pathway.

Summarily, these results indicate that the transcriptional activity of ABCA1 is enhanced by the transcription factor PREB, which is a downstream of the CaMKK/CaMKIV signaling pathway stimulated by exendin-4.

### 3.6. Exendin-4 increases the expression of ABCA1 and decreases lipid accumulation in the liver of mice with a HFD

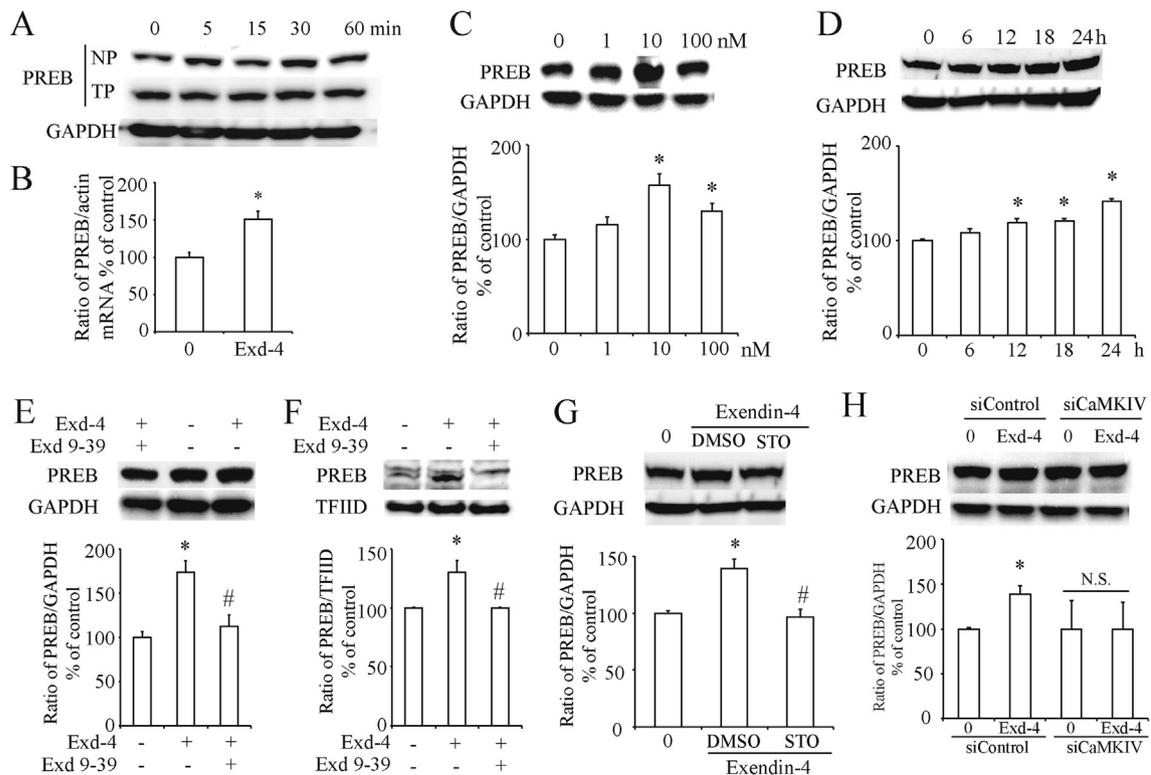
To verify the effect of exendin-4 *in vivo*, we injected exendin-4 at 1 nmol/kg/day into wild type (WT) mice fed on a HFD or a NFD. Exendin-4 increased protein and mRNA expressions of ABCA1 as well as the protein level of PREB in the liver of HFD-fed mice (Figure 7A,B) without affecting the expression of ABCG1 (Figure S3). Similar to the results *in vitro*, treatment with exendin-4 significantly decreased lipid accumulation in the liver of HFD-fed mice (Figure 7C,D), whereas it had almost no effect on NFD-fed mice (Figure S4 and S5). At the same time, treatment with exendin-4 significantly decreased body weight gain (Figure 8A and Figure S6A), high level of fasting blood glucose (Figure 8B), total cholesterol (Figure 8C), low density lipoprotein-cholesterol (LDL-C) (Figure 8E), and TG (Figure 8F) in HFD mice without affecting the level of HDL-cholesterol (HDL-C) (Figure 8D). Notably, treatment with exendin-4 also decreased the level of TG in WT mice with NFD. These results confirmed the role of

exendin-4 in protecting the liver from cholesterol accumulation *in vivo*.

We have demonstrated *in vitro* that PREB could regulate the expression of ABCA1 and cholesterol content in hepatocytes. *In vivo*, we found that HFD treatment significantly decreased the expression of hepatic PREB in WT mice (Figure 7E,F). Therefore, we overexpressed PREB to establish PREB-transgenic (PREB-Tg) mice and verified the effect of PREB *in vivo*. In the HFD groups, PREB-Tg mice had a higher protein and mRNA levels of ABCA1 (Figure 7G,H), as well as lower cholesterol content (Figure 7I) and fewer lipids in the liver (Figure 7D and Figure S7) compared with WT mice, whereas overexpression of PREB has no effect on the expression of ABCG1. Consistently, the overexpression of PREB dramatically decreased the high levels of total cholesterol and LDL-C induced by HFD (Figure 8C,E). PREB seemed to decrease the body weight gain and serum TG level in both the NFD and HFD groups (Figure 8A,F; Figure S6B and C), whereas it had no effect on fasting blood glucose and HDL-C (Figure 8B,D). These results pointed to the role of PREB against cholesterol accumulation induced by a HFD.

## 4. DISCUSSION

Accumulation of lipid in the liver induces fatty liver disease. Non-alcoholic fatty liver disease (NAFLD) is one of the most common diseases, affecting 20%–30% of adults in developed countries [34]. Accumulating evidences proved that NAFLD is highly associated with metabolic syndromes, such as type 2 diabetes (T2D); and, the prevalence of NAFLD in patients with T2D is estimated to be approximately



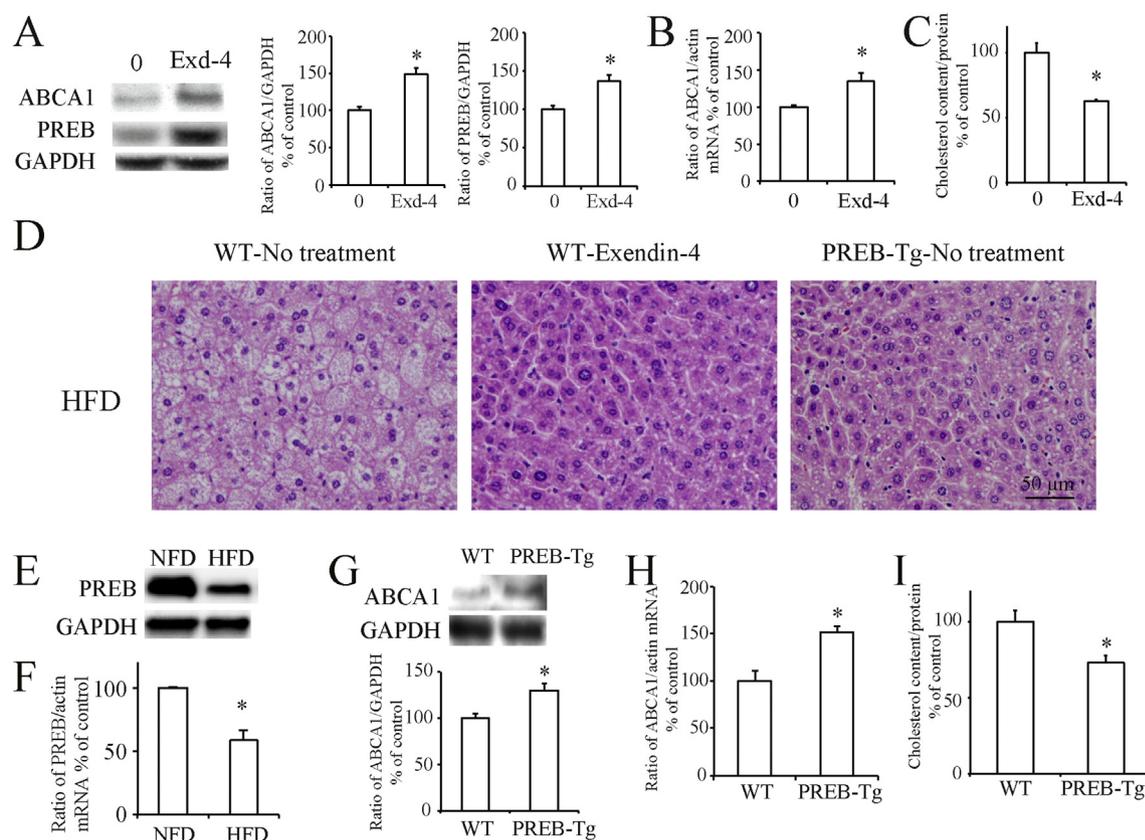
**Figure 6:** Effects of exendin-4 on the expression of PREB in HepG2 cells. A, Nuclear and total expression of PREB in HepG2 cells treated with exendin-4 for a short time. B, Effect of exendin-4 on PREB mRNA expression. C and D, Effect of exendin-4 on PREB expression. E and F, Total and nuclear expression of PREB in HepG2 cells treated with exendin-4 or exendin-9-39. G, Effect of STO-609 on PREB expression. H, Effect of exendin-4 on PREB expression after silencing of CaMKIV. A graph shows the mean  $\pm$  SEM ( $n = 3$ ) of separate group. Exd-4, exendin-4; Exd9-39, exendin9-39; STO, STO-609. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with exendin-4; N.S., not significant.

60% [35,36]. A recent study suggested that GLP-1R agonists for T2D may become an alternative treatment option for NAFLD [6]. Moreover, exendin-4, one of the GLP-1R agonists, reversed hepatic steatosis in *ob/ob* mice [9,10]. In this study, we checked the underlying molecular mechanism, and our results showed that both *in vitro* and *in vivo*, exendin-4 improved fatty liver disease via the upregulation of cholesterol transporter ABCA1, which is mediated by the CaMKK/CaMKIV/PREB signaling pathway.

ABCA1 is a membrane protein that is required for HDL formation to facilitate cholesterol efflux. In Tangier disease, the absence of ABCA1 induces cholesterol accumulation in peripheral tissue and causes severe HDL deficiency [11]. Typically, specific ABCA1 knockout in hepatocytes decreased the generation of heterogeneous-sized nascent HDL particles *in vivo*, resulting in large TG-rich very low density lipoprotein (VLDL) accumulation in the liver [37]. Thus, ABCA1 has a beneficial effect to protect the liver from cholesterol accumulation. In our study, we demonstrated that the upregulation of hepatic ABCA1 by exendin-4 contributed to the reduction in lipid accumulation in the liver and the high level of plasma total cholesterol and TG induced by a HFD. Recently, hepatic ABCA1 expression has been demonstrated to protect pancreatic insulin secretion and to improve glucose homeostasis in diabetes [38]. This is confirmed by our *in vivo* experiments, which showed that treatment with exendin-4 upregulated hepatic ABCA1 and reduced fasting blood glucose level in HFD-induced hyperglycemic mice (Figure 8). Also, ABCA1 expression in pancreatic beta cells has been proved to protect insulin secretion against lipotoxicity induced by cholesterol accumulation [14,39]. These studies suggest that the

upregulation of ABCA1 may serve as a novel therapeutic target in diabetes and fatty liver disease.

As a novel potential treatment of NAFLD, GLP-1R agonists showed beneficial effects on decreasing hepatic TG associated with plasma aminotransferases in T2D [6,36,40]. A recent small early clinical trial in 52 patients with nonalcoholic steatohepatitis (NASH) with or without T2D showed that resolution of NASH occurred in 9/23 patients after 48 weeks of liraglutide, as one of the most widely studied GLP-1R agonists, whereas resolution occurred in 2/22 patients in the placebo group ( $p = 0.019$ ) [5]. Semaglutide, a novel long-acting GLP-1R agonist with a half-life of 165 h, was also proved to decrease cardiovascular events in patients with T2D [41,42], and an ongoing clinical trial (NCT02970942) is investigating its potential for treating NASH. Exendin-4 (exenatide) has been demonstrated clinically to protect liver function and the cardiovascular system [6,43]. Compared with metformin, exendin-4 has a more significant effect on improving liver enzymes [44]. These studies pointed to the important role of GLP-1R agonists in improvement of liver function in NAFLD with T2D. In mice fed a HFD, acute activation of GLP-1R decreases TG content in the liver [45], whereas 2-week treatment with GLP-1 decreases plasma TG level [46]. These findings are consistent with our results, which showed that exendin-4 significantly decreased the levels of lipid content in the hepatocytes, plasma total cholesterol, LDL-C, and TG in HFD-fed mice. The idea of GLP-1R expression in hepatocytes was first raised in 2006, and it was able to reverse hepatic steatosis in *ob/ob* mice [9]. Because mature antibody is unable to recognize GLP-1R protein and to detect the full length of GLP-1R mRNA, the expression of GLP-1R in hepatocytes is controversial [47]. To better



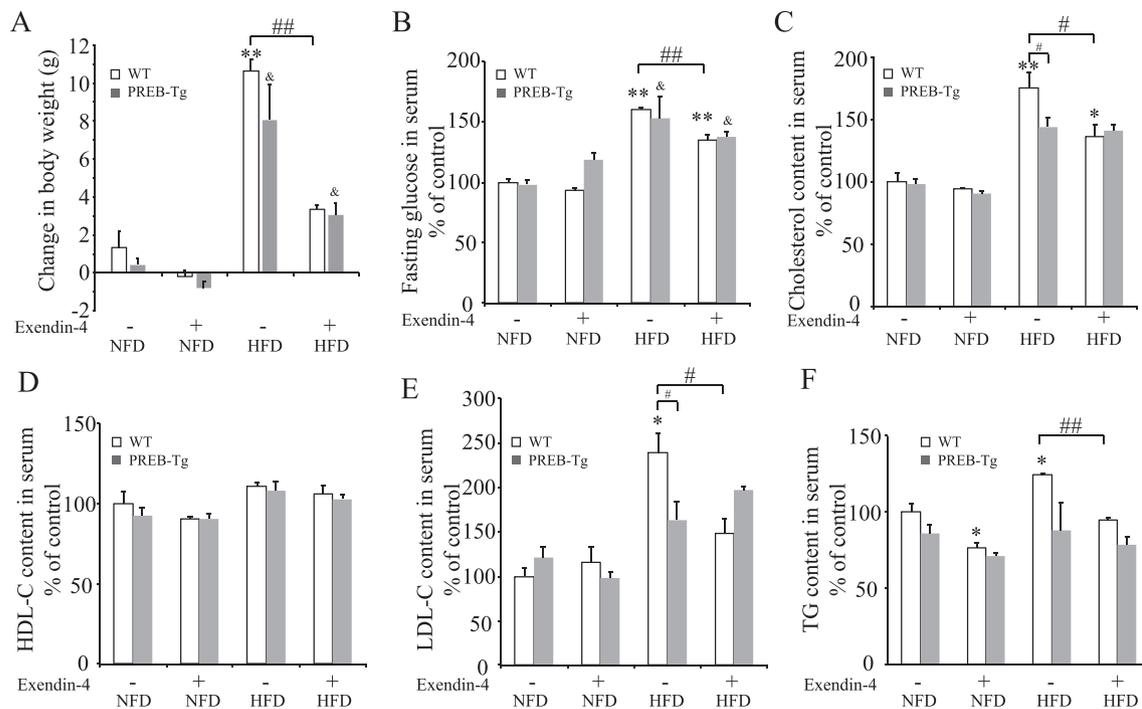
**Figure 7:** The role of exendin-4 and PREB in lipid accumulation in the liver of mice. In the liver of mice fed a high-fat diet (HFD), effect of exendin-4 on ABCA1 and PREB expression (A and B), and cholesterol content (C). D, H&E stain of liver from wild type (WT) or PREB-transgenic (PREB-Tg) mice. E and F, protein and mRNA level of hepatic PREB in WT mice fed a normal food diet (NFD) or a HFD. In WT and PREB-Tg mice fed a HFD, protein and mRNA level of hepatic ABCA1 (G and H), and cholesterol content (I). Each data point shows the mean  $\pm$  SEM ( $n \geq 3$ ) of separate treatment. \* $P < 0.05$  compared with control. Exd-4 or Exendin-4, injection of exendin-4 at 1 nmol/kg/day for 4 weeks.

understand the function of GLP-1R in hepatocytes, we used its antagonist, exendin9-39, to inhibit the activation of GLP-1R. Treatment with exendin9-39 cancelled all effects of exendin-4 on the upregulation of hepatic ABCA1 and the reduction in cholesterol content, suggesting that GLP-1R may be essential in regulating ABCA1 expression and lipid homeostasis. Although GLP-1R agonists exhibited their benefits on improving NAFLD in patients with T2D, currently, they are still not recommended by the American Association for the Study of Liver Disease (AASLD) and the European Association for the Study of Liver (EASL) to treat NAFLD without diabetes because of insufficient evidences [48,49]. Our current results may provide a basic knowledge on the effectiveness of GLP-1R agonists in treating NAFLD.

Our previous study indicated that exendin-4 was able to stimulate the CaMKK/CaMKIV signaling pathway to induce ABCA1 expression in pancreatic beta cells [50]. Therefore, we identified the role of CaMKK and CaMKIV in the upregulation of ABCA1 by exendin-4 in the present study. Inhibition of CaMKK by its specific inhibitor, STO-609, markedly reversed the effect of exendin-4 on the induction of ABCA1 expression and induced cholesterol accumulation in HepG2 cells. This is in agreement with a recent report that the phosphorylation of CaMKK contributes to the decrease in lipid content in the liver of mice fed a HFD [51]. As a downstream of CaMKK, CaMKIV is phosphorylated to translocate from the cytoplasm to the nucleus [32] and takes part in regulating gene expression. Consistently, the activation of CaMKIV at Thr196 site and nuclear recruitment stimulated by exendin-4 are also indicated in our study. Furthermore, silencing of CaMKIV abrogated the

effect of exendin-4 on the induction of ABCA1 expression. These results showed the important role of CaMKK/CaMKIV in regulating hepatic ABCA1 and ameliorating cholesterol accumulation in the hepatocytes. Downregulation of CaMKIV is found in the pancreatic beta cells with glucotoxicity induced by high glucose, suggesting that CaMKIV may be involved in glucose metabolism and the pathology of diabetes [52]. Recently, CaMKIV was reported as an essential regulator of hepatic cancer; and, the activation of CaMKIV by CaMKK2 is considered to induce hepatocellular carcinoma [53]. Thus, the role of the CaMKK/CaMKIV pathway in regulating liver function needs to be further studied.

Several studies have shown that nuclear CaMKIV can phosphorylate a certain transcription factor and mediate transcriptional stimulation through cAMP-responsive element-binding (CREB) protein phosphorylation [54,55]. In addition, CaMKIV enhances the activity of the activating transcription factor 2, which binds to human insulin gene for cAMP-responsive elements [56]. Therefore, we searched for a cAMP-responsive element (5'-TGAC-3') in the ABCA1 promoter region to find the transcription binding protein that can regulate ABCA1 gene transcription. Unfortunately, there was no cAMP-responsive element in the human ABCA1 promoter region. However, we found a novel transcription factor, prolactin regulatory element binding (PREB), which is also responsive to cAMP stimulation to regulate gene transcription [20]. By ChIP assay, we confirmed that PREB could directly bind to the ABCA1 promoter region, and this binding was enhanced by exendin-4. Moreover, we demonstrated that PREB was regulated by the CaMKK/



**Figure 8:** Parameters in wide type (WT) or PREB-transgenic (PREB-Tg) mice treated with or without exendin-4. Wild type (WT) or PREB-Tg mice were fed on normal food diet (NFD) or a high-fat diet (HFD) for 8 weeks and injected with exendin-4 from the 5th week. A, Change in body weight from the 5th week to the 8th week. B, Fasting glucose in serum at the 8th week. C–F, Plasma level of total cholesterol, HDL-C, LDL-C, TG at the 8th week. Each data point shows the mean  $\pm$  SEM ( $n \geq 3$ ) of separate treatment. \* or \*\* $P < 0.05$  or  $P < 0.01$  compared with WT mice fed a NFD; &  $P < 0.05$  compared with PREB-Tg mice fed a NFD; # or ## $P < 0.05$  or  $P < 0.01$  compared with WT mice fed a HFD.

CaMKIV pathway, which is similar to the role of CREB as a transcription factor [55]. In addition, our data showed that overexpression of PREB markedly enhanced ABCA1 promoter activity in HepG2 cells, whereas mutation of the PREB binding site (5'-TGAT-3') in the ABCA1 promoter region or the silencing of PREB abrogated the effect of exendin-4 on the upregulation of ABCA1. These observations are consistent with our previous studies showing that exendin-4 stimulates pancreatic ABCA1 promoter activity by the CaMKK/CaMKIV signaling pathway and the transcription factor PREB [18,19].

*In vitro*, we demonstrated that the silencing of PREB induced cholesterol accumulation and cancelled the effect of exendin-4 on the reduction of cholesterol content in HepG2 cells, pointing out the essential role of PREB in improving hepatic cholesterol accumulation. To further study the role of PREB, we generated PREB-transgenic (PREB-Tg) mice, in which PREB expression is upregulated [24]. Our data showed that PREB-Tg mice fed a HFD had lower hepatic cholesterol content and plasma cholesterol, especially lower plasma LDL-C compared with wild type mice. The expression of hepatic ABCA1 significantly increased in PREB-Tg mice. In addition, a HFD decreased the expression of hepatic PREB and induced cholesterol accumulation in wild type mice, suggesting that PREB may serve as a key regulator in protecting the liver from fatty liver disease. Our data are in agreement with recent research that PREB is downregulated in *db/db*, *ob/ob*, and HFD-treated mice [57]. They also showed that the reduction of PREB correlated with the upregulation of the hepatic gluconeogenic gene and impaired tolerance of glucose, insulin, and pyruvate, which are improved by the administration of adenovirus-PREB [57]. Consistently, our results showed that a HFD induced a higher level of fasting blood glucose with the reduction of hepatic PREB in wild type mice. However, high fasting blood glucose induced by a HFD was not ameliorated in

PREB-Tg mice. Moreover, PREB has proved to be involved in hepatitis C virus (HCV) replication, and HCV infection induces the expression of PREB in hepatocytes [58]. Further research is needed to investigate the role of PREB in hepatic lipid metabolism. PREB is also highly expressed in many human tissues, and our previous study showed PREB mediated the monocyte chemoattractant protein-1 (MCP-1) transcription induced by cytokines in human endothelial cells, which may contribute to the inflammation process in the cardiovascular system [59]. However, more studies are needed to assess the role of PREB in improving human NAFLD, because the HFD mouse model to induce NAFLD displays early disease stages and exhibits slightly less severe NASH pathology than in humans [60,61].

In summary, our *in vitro* and *in vivo* findings have shown that exendin-4 suppresses cholesterol accumulation in the hepatocytes via elevation of hepatic ABCA1, which is mediated by the CaMKK/CaMKIV/PREB signaling pathway (Figure S8). This finding suggests that the use of exendin-4 in diabetes has a potential role in protecting the liver from fatty liver disease, and the transcription factor PREB and ABCA1 may be the therapeutic targets in fatty liver disease.

#### AUTHOR CONTRIBUTIONS

Jingya Lyu: conceived the study, conducted the experiments, collected and analyzed data, and wrote/revised the manuscript. Hitomi Imachi, Kensaku Fukunaga, Seisuke Sato, Toshihiro Kobayashi, Tao Dong, Takanobu Saheki and Mari Matsumoto: conceived the study and analyzed data. Hisakazu Iwama: searched the potential binding of transcription factors in ABCA1 promoter. Huanxiang Zhang and Koji Murao: conceived the study, received the funding and reviewed the manuscript.

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## CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2019.12.015>.

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