

# Osteopontin deficiency protects mice from cholesterol gallstone formation by reducing expression of intestinal NPC1L1

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**Abstract.** Homeostasis of cholesterol is regulated by absorption in the intestine and synthesis in the liver. The authors previously demonstrated that OPN (osteopontin) exhibits the ability to alter hepatic cholesterol metabolism, thus affecting cholesterol gallstone formation in mice. The present study investigated the role of OPN in cholesterol gallstone formation, focusing on its effect on intestinal absorption of cholesterol. OPN gene knockout (OPN<sup>-/-</sup>) mice and wild-type mice were respectively fed with a chow or lithogenic diet (LD) for 8 weeks. Following an 8-week LD period, the incidence of gallstone, bile composition, level of serum and fecal lipids and the expression of intestinal associated genes were analyzed. OPN<sup>-/-</sup> mice were protected from gallstone formation induced by 8 weeks' LD-feeding. This protective effect from OPN deficiency was associated with alterations in bile composition, including a reduced concentration of biliary cholesterol. Additionally, plasma cholesterol level was decreased in LD-fed OPN<sup>-/-</sup> mice. The alterations primarily resulted from the decreased expression of intestinal Niemann-Pick C1-like (NPC1 L) 1, which is important in the intestinal absorption of cholesterol. The present study demonstrated that OPN deficiency reduced intestinal

absorption of cholesterol by suppressing the expression of NPC1L1, thus protecting mice from cholesterol gallstone formation.

## Introduction

Gallstone disease is a global major health problem, and its attendant complications and comorbidities impose a huge financial burden on health care economy (1-4). Gallstone disease is a disease with multiple factors, mainly caused by the complex interaction of genetic factors and environmental factors (5). A prerequisite for cholesterol gallstone formation is biliary precipitation of excess cholesterol as solid crystals (6,7). The most important source of biliary cholesterol is preformed plasma lipoprotein cholesterol which is mainly absorbed from the diet in small intestine, whereas only a minor contribution of cholesterol is generated by hepatic new synthesis or hydrolysis of cholesteryl ester stores (8). Dietary cholesterol is trafficked into enterocyte by Niemann-Pick C1-like 1 (NPC1L1) protein (9), and can be reversely transported to the intestinal lumen by ATP-binding cassette, sub-family G, member 5/8 (ABCG5/8) (10,11). After cholesterol passes through the enterocyte membrane, it should be esterified by acetyl-Coenzyme A acetyltransferase 2 (ACAT2); subsequent to this, it is transported in the chylomicrons circulating then reach the liver for biliary secretion (12,13). Therefore, a high fat intake increases the risk of developing gallstone. And inhibiting NPC1L1 expression suppresses intestinal absorption of cholesterol, reduces plasma cholesterol level and prevents the development of gallstone (14).

Osteopontin (OPN) is a soluble cytokine and a matrix-associated protein presenting in the majority of tissues and body fluids (15). Previous study found that plasma OPN level had a significant correlation with plasma cholesterol concentration in human (16). Our previous study demonstrated that OPN was involved in the pathogenesis of gallstone disease (17,18), and that OPN altered hepatic cholesterol metabolism thus affecting cholesterol gallstone formation in mice (19). Because the homeostasis of cholesterol is regulated by both absorption in intestine and synthesis in liver (20,21), it is also important to discover the role of OPN in intestinal cholesterol absorption. Therefore, we investigated the mechanism

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*Abbreviations:* OPN, osteopontin; OPN<sup>-/-</sup>, OPN gene knockout; WT, wild-type; CD, chow diet; LD, lithogenic diet; NPC1L1, Niemann-Pick C1-like L1; ABCG5/8, ATP-binding cassette, sub-family G, member 5/8; ACAT2, acetyl-Coenzyme A acetyltransferase 2; ASBT, apical sodium-dependent bile acid transporter; ILBP, ileal lipid binding protein; OSTα/β, organic solute transporter α/β; ABCA1, ATP-binding cassette, sub-family A, member 1; LXRα, liver X receptor α; PPARδ, peroxisome proliferator activated receptor δ; CSI, cholesterol saturation index; SD, standard deviation

*Key words:* cholesterol, gallstone formation, mice, Niemann-Pick C1-like L1, osteopontin

of OPN in cholesterol gallstone formation focusing on its effect on intestinal absorption of cholesterol in this study.

## Materials and methods

**Animals, diets and sample collection.** WT mice were purchased from Fudan University (Shanghai, China). OPN<sup>-/-</sup> mice in congenic background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed at 22±2°C and 60±10% relative humidity in a specific pathogen-free environment, with a 12:12 h light: dark cycle. WT and OPN<sup>-/-</sup> male mice between 8 and 10 weeks of age were fed a chow diet (CD) or LD (CD supplemented 15% fat, 2% cholesterol, and 0.5% cholic acid) for up to 8 weeks. Bile was collected, and crystal analysis was immediately performed. Blood was collected via right ventricle heart puncture. Feces was collected from individually housed mice for 72 h. The tissues were harvested and then snap-frozen in liquid nitrogen for protein and RNA isolation or fixed in 4% paraformaldehyde overnight for histological analysis. All animals received humane care and all experimental procedures were conducted in conformance with principles of the National Institutes of Health guide for the care and use of laboratory animal (NIH Publications No. 8023) and approved by the Animal Ethics Committee of Fudan University.

**Gallstone formation analysis and biochemical detections of bile, serum and feces.** Gallstone were defined as macroscopically visible stones, whereas crystals were defined with polarizing light microscopy (Olympus, Tokyo, Japan). The fecal lipids and bile acids were extracted as previously described (8,22). Cholesterol, bile acids and triglyceride levels were measured with assay kits from KHB (Shanghai, China), and phospholipid content was measured using an assay kit from Wako (Osaka, Japan) according to the manufacturers' instructions. All the biochemical measures were assayed in triplicate 3 times. Then the cholesterol saturation index (CSI) was calculated according to Carey's critical tables (23).

**Quantitative real-time PCR analysis.** Total RNAs were collected from the proximal segment of small intestine which was equally randomly divided into 5 segments or liver in each mouse with the RNAPrep Pure Tissue kit (TianGen, Beijing, China) according to the manufacturer's instructions. Random primers (Takara, Shiga, Japan) were used for reverse transcription of total RNA to complementary DNA. Quantitative real-time PCR was performed using SYBR-Green I chemistry (TianGen) and the ABI 7900HT Fast Real-Time PCR System (Applied Biosystem, Shanghai, China). The gene-specific primer sequences are shown in Table I. Messenger RNA (mRNA) expression levels were calculated relative to the housekeeping gene GAPDH and further normalized to the expression levels of the respective controls following the basis of the relative expression method (24).

**Western blot analysis.** The entire proximal segment of small intestine that was randomly divided into 5 equal segments were lysed in ice-cold RIPA buffer supplemented with protease inhibitors. Protein concentration of the extracts was measured by the BCA Protein Assay kit (Thermo, Shanghai, China). The

protein was analyzed by 8% SDS-PAGE and transferred to Poly (vinylidene fluoride) membranes. Anti-NPC1L1 antibody (Santa Cruz, Shanghai, China) was used at a 1:300 dilution. A 1:10,000 dilution of rabbit anti-goat immunoglobulin G-HRP (Santa Cruz) was used as a secondary antibody. After probing individual antibodies, the antigen-antibody complex was visualized by Enhanced Chemiluminescence Reagents Supersignal (TianGen, Beijing, China). The relative average protein level was determined by densitometry using Image J software (NIH, Maryland, and USA).

**Histopathological and immunohistochemistry analysis.** Tissues were fixed in 4% paraformaldehyde overnight and then transferred to 70% ethanol prior to routine processing and staining with hematoxylin and eosin. Formalin-fixed tissues were embedded in paraffin and cut into 3 mm sections. Sample slides were incubated with anti-NPC1L1 antibodies (Santa Cruz) with a 1:75 dilution, followed by 30 min incubation with an HRP-labeled polymer secondary antibody (Santa Cruz). Sections were viewed with a Nikon ECLIPSE E600 microscope (Nikon, Tokyo, Japan) using x10 objective lenses, and images were acquired with a SPOT INSIGHT™ digital color camera, model 3.2.0 (Sterling, Heights, MI). Quantification of immunoreactivity was performed on digitally captured color images saved as TIFF files and analyzed using Image-Pro plus 6.0 software (Media Cybernetics, Florida, USA). The blind histopathological and immunohistochemistry analysis were conducted by a pathologist of Fudan University.

**Statistical analysis.** Data are demonstrated as mean ± standard deviation (SD) unless otherwise noted. The statistical significance of differences between the means of the experimental groups was evaluated with unpaired Student's t test (GraphPad, La Jolla, CA, USA). A difference was considered to be statistically significant at P<0.05.

## Results

**OPN deficiency reduced gallstone formation in LD-fed mice.** Neither WT mice (0/6) nor OPN<sup>-/-</sup> mice (0/6) showed crystals or gallstones when fed a CD. When fed with LD for 8 weeks, all WT mice (6/6) developed gallstones, whereas the penetrance in OPN<sup>-/-</sup> mice was 16.7% (1/6). Compared to those of OPN<sup>-/-</sup> mice, the gallbladder bile of WT mice appeared turbid and full of precipitates and stones. Microscopic examination of the gallbladder bile revealed cholesterol crystals in WT mice, whereas those of OPN<sup>-/-</sup> mice were largely free of cholesterol precipitates (Fig. 1).

**OPN deficiency altered the cholesterol content and CSI in LD-fed mice.** The histology examinations of gallbladder and ileum were similar in WT mice and OPN<sup>-/-</sup> mice (Fig. 2). And the body weight and gallbladder volume between two strains showed no difference (Table II). The cholesterol content of two genotypes mice were also similar when fed CD. After being fed with LD for 8 weeks, OPN<sup>-/-</sup> mice exhibited reduced in biliary cholesterol content and serum cholesterol profile compared to WT mice. But the fecal cholesterol content showed no statistically difference in two LD-fed strains (Table II). There was no difference in serum phospholipid, triglyceride and bile acids

Table I. Primer sequences for quantitative real-time PCR in mice.

Gene	Sense primer (5'→3')	Antisense primer (5'→3')
NPC1L1	CTCTGCCCTCTGCAATGCTC	GAACAGGCTGCCGAGTCTT
ABCG5	AGGGCCTCACATCAACAGAG	GCTGACGCTGTAGGACACAT
ABCG8	TGCCACCTTCCACATGTC	ATGAAGCCGGCAGTAAGGTAGA
ACAT2	CTACAAGCAAGACCCAAGAG	CATGTGGTAGATGGTTCGG
ABCA1	CGTTTCCGGGAAGTGTCCTA	GCTAGAGATGACAAGGAGGATGGA
LXR $\alpha$	GATAGGGTTGGAGTCAGCA	GGAGCGCCTGTTACACTGTT
PPAR $\delta$	TCCATCGTCAACAAAGACGGG	ACTTGGGCTCAATGATGTCAC
OST $\alpha$	AGGCAGGACTCATATCAAACCTTG	TGAGGGCTATGTCCACTGGG
OST $\beta$	AGATGCGGCTCCTTGGAAATTA	TGGCTGCTTCTTTTCGATTTCTG
ASBT	GTCTGTCCCCCAAATGCAACT	CACCCCATAGAAAACATCACCA
ILBP	CTTCCAGGAGACGTGATTGAAA	CCTCCGAAGTCTGGTGATAGTTG
HMGS	GCCGTGAACTGGGTCGAA	GCATATATAGCAATGTCTCCTGCAA
HMGR	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT
SREBP2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
LDLR	TGGCTGTTCCCACATCTG	CTCGTCAATATCTTCACACCTG
LRP	ACTATGGATGCCCTAAAACCTTG	GCAATCTCTTTCACCGTCACA
PCSK9	ACCCTCATAGGCCTGGAGTT	CTGTGATGACCTCTGGAGCA
GAPDH	TGTGTCGGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT

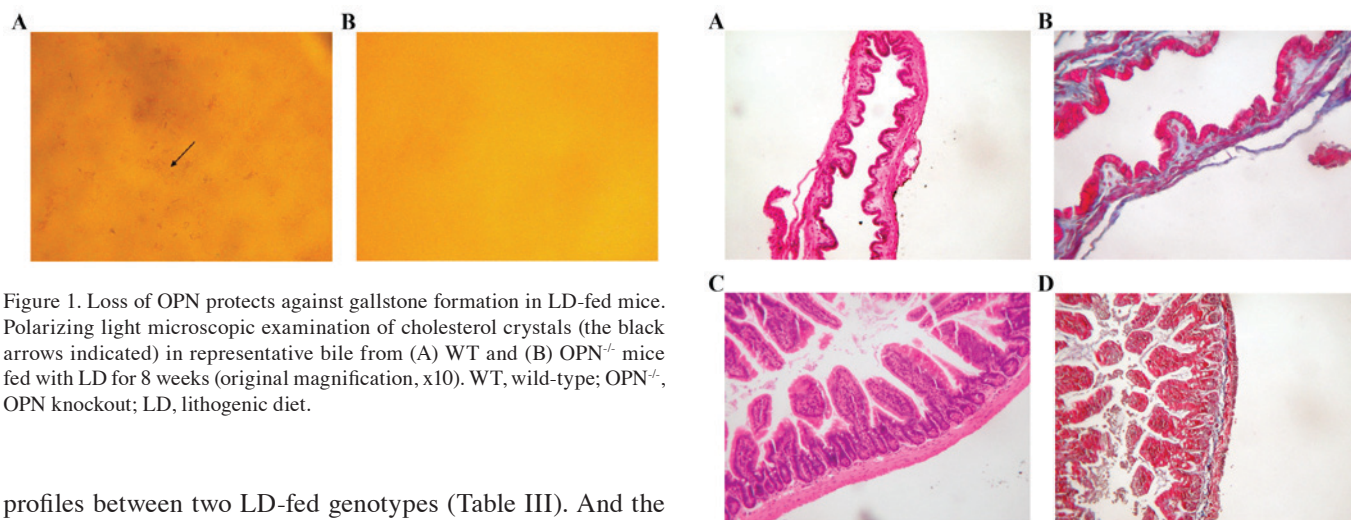


Figure 1. Loss of OPN protects against gallstone formation in LD-fed mice. Polarizing light microscopic examination of cholesterol crystals (the black arrows indicated) in representative bile from (A) WT and (B)  $OPN^{-/-}$  mice fed with LD for 8 weeks (original magnification, x10). WT, wild-type;  $OPN^{-/-}$ , OPN knockout; LD, lithogenic diet.

profiles between two LD-fed genotypes (Table III). And the fecal bile acids content showed no statistically difference in two strains. However, the LD-fed  $OPN^{-/-}$  mice increased in biliary bile acids and biliary phospholipid content compared to LD-fed WT mice (Table III). This combined effect of biliary biochemical alterations led to a decreased CSI in LD-fed  $OPN^{-/-}$  mice (Table II), providing a biochemical mechanism for the phenotype that protecting  $OPN^{-/-}$  mice from cholesterol gallstone formation.

*OPN deficiency suppressed the expression of intestinal NPC1L1 in LD-fed mice.* To understand the mechanisms responsible for the changes in biochemical contents, we profiled the expression of intestinal related genes. There was no significant difference in genes expression between the two genotypes on CD (Fig. 3A and B). When challenged with the LD for 8 weeks,  $OPN^{-/-}$  mice showed no difference in the expression of apical sodium-dependent bile acid transporter (ASBT), ileal lipid binding protein (ILBP) and organic solute

Figure 2. Histology examinations of gallbladder and ileum in WT and  $OPN^{-/-}$  mice. Histology examinations of gallbladder from (A) WT and (B)  $OPN^{-/-}$  mice fed with LD for 8 weeks (original magnification, x200). Histology examinations of ileum from (C) WT and (D)  $OPN^{-/-}$  mice fed with LD for 8 weeks (original magnification, x200). WT, wild-type;  $OPN^{-/-}$ , OPN knockout; LD, lithogenic diet.

transporters  $\alpha/\beta$  (OST $\alpha/\beta$ ), which are involved in intestinal reabsorption of bile acids (Fig. 3C). Among the intestinal cholesterol transporters, the expression of NPC1L1 had an almost 50% reduction in LD-fed  $OPN^{-/-}$  mice, whereas the expression of ABCG5/8, ATP-binding cassette, sub-family A, member 1 (ABCA1), and ACAT2 was not affected (Fig. 3D). Then we detected the gene expression of liver X receptor  $\alpha$  (LXR $\alpha$ ) and peroxisome proliferator activated receptor  $\delta$  (PPAR $\delta$ ), and found it was similar between two mice strains (Fig. 3D). Then we challenged the protein expression of

Table II. The body weight, gallbladder volume and cholesterol content in WT and OPN<sup>-/-</sup> mice.

Parameters	CD		LD for 8 weeks	
	WT	OPN <sup>-/-</sup>	WT	OPN <sup>-/-</sup>
Body weight (g)	20.1±1.06	20.5±1.06	20.3±0.75	22.8±2.38
Gallbladder volume (μl)	23.2±7.57	25.6±11.5	27.7±9.14	23.5±6.51
Biliary cholesterol (mmol/l)	2.58±0.67	2.88±0.59	8.81±1.14	5.34±0.90 <sup>a</sup>
Serum cholesterol (mmol/l)	1.85±0.15	1.96±0.33	3.10±0.56	2.31±0.25 <sup>a</sup>
Fecal cholesterol output (mg/g feces/day)	3.83±0.84	3.77±0.73	15.29±1.22	17.08±2.11
CSI	0.48±0.12	0.49±0.05	1.19±0.13	0.59±0.07 <sup>b</sup>

The data are expressed as mean ± SD (n=6 per groups). Statistical analysis was performed by unpaired Student's t test, <sup>a</sup>P<0.05, <sup>b</sup>P<0.01. WT, wild-type; OPN<sup>-/-</sup>, OPN knockout; CD, chow diet; LD, lithogenic diet; CSI, cholesterol saturation index.

Table III. Lipids and bile acids profiles in WT and OPN<sup>-/-</sup> mice.

Parameters	CD		LD for 8 weeks	
	WT	OPN <sup>-/-</sup>	WT	OPN <sup>-/-</sup>
Biliary bile acids (mmol/l)	56.65±6.08	63.59±7.66	79.79±11.0	97.34±15.8 <sup>a</sup>
Biliary phospholipid (mmol/l)	20.53±1.55	21.99±2.82	24.68±2.45	30.48±4.80 <sup>a</sup>
Serum phospholipid (mg/dl)	157.4±18.1	167.0±31.0	182.6±61.0	190.7±22.5
Serum bile acids (μmol/l)	4.30±5.15	4.17±3.13	10.3±8.03	13.5±5.25
Serum triglyceride (mmol/l)	0.32±0.19	0.39±0.18	0.46±0.73	0.30±0.03
Fecal bile acids output (μmol/g feces/day)	2.82±0.25	3.17±0.75	66.80±5.21	71.3±11.80

The data are expressed as mean ± SD (n=6 per groups). Statistical analysis was performed by unpaired Student's t test, <sup>a</sup>P<0.05, <sup>b</sup>P<0.01. WT, wild-type; OPN<sup>-/-</sup>, OPN knockout; CD, chow diet; LD, lithogenic diet.

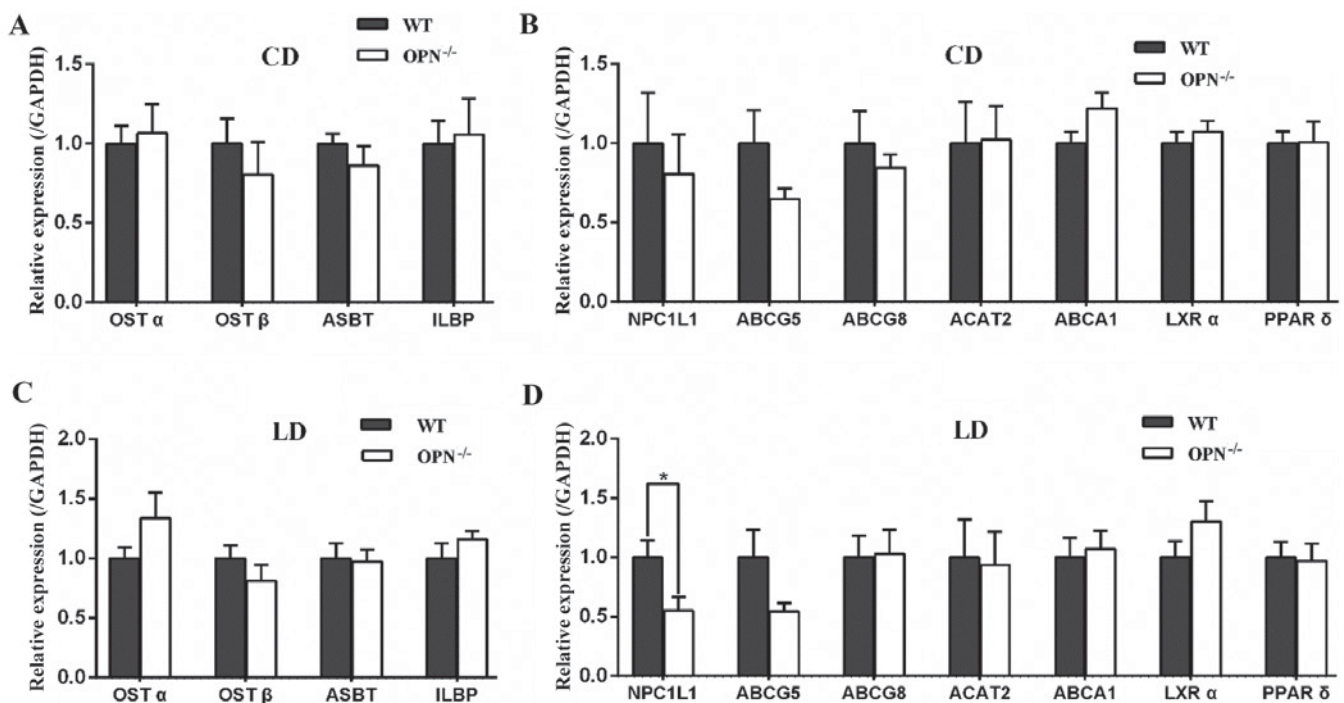


Figure 3. The mRNA expression of intestinal related genes in WT and OPN<sup>-/-</sup> mice. Quantitative real-time PCR analysis of mRNA levels for intestinal related genes from WT mice and OPN<sup>-/-</sup> mice fed a (A and B) CD or (C and D) LD for 8 weeks. The data are expressed as the mean ± SD (n=6 per group). Statistical analysis was performed by unpaired Student's t test, <sup>a</sup>P<0.05. WT, wild-type; OPN<sup>-/-</sup>, OPN knockout; CD, chow diet; LD, lithogenic diet.

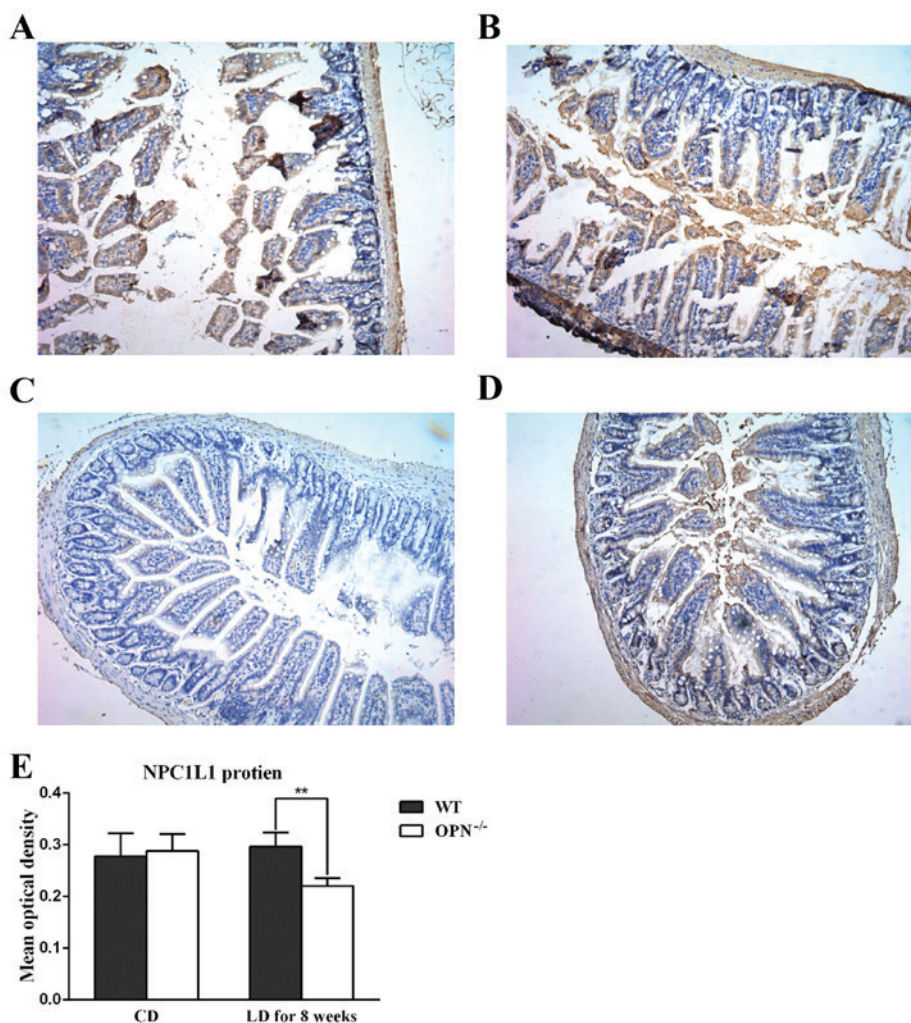


Figure 4. Immunohistochemistry analysis of NPC1L1 protein expression in mice. Ileum specimens from (A) CD-fed OPN<sup>-/-</sup>, (B) CD-fed WT, (C) LD-fed OPN<sup>-/-</sup>, (D) LD-fed WT mice that were immunohistochemically stained for NPC1L1. (E) Quantitative immunohistochemical analysis of ileum NPC1L1 levels by mean optical density in mice. The data are expressed as the mean  $\pm$  SD (n=6 per group). Statistical analysis was performed using unpaired Student's t test, \*\*P<0.01. WT, wild-type; OPN<sup>-/-</sup>, OPN knockout; CD, chow diet; LD, lithogenic diet.

intestinal NPC1L1 with immunohistochemistry, and found that the protein expression of NPC1L1 was similar in two CD-fed strains but decreased in LD-fed OPN<sup>-/-</sup> mice (Fig. 4). And the changed expression of NPC1L1 protein was also confirmed by western blot analysis (Fig. 5).

Because the homeostasis of cholesterol is regulated by both absorption in intestine and synthesis in liver, we also measured the expression of hepatic related genes. There were no significant differences in those genes between the two genotypes fed a CD (Fig. 6A). When challenged with the LD for 8 weeks the expression of proprotein convertase subtilisin/kexin type 9 (PCSK9) tended to be decreased in OPN<sup>-/-</sup> mice, but the change was not statistically significant. The expression of other hepatic related genes also showed no difference between two mice strains fed a LD (Fig. 6B).

## Discussion

In this study, we found that OPN deficiency could attenuate the absorption of cholesterol by reducing intestinal expression of NPC1L1, thus protecting mice from diet-induced cholesterol gallstone formation.

OPN<sup>-/-</sup> mice was less susceptible to diet-induced gallstone than WT mice, which is consistent with our previous work (19). The body weight and gallbladder volume did not differ between the OPN<sup>-/-</sup> mice and WT mice fed with CD nor LD for 8 weeks. In addition, gallbladder histology was also similar in two strains. These findings indicate that the abnormalities of gallbladder are unlikely to be primarily responsible for the protection of LD-fed OPN<sup>-/-</sup> mice from gallstone formation. As the prerequisite for cholesterol gallstone formation is the precipitation of excess cholesterol in bile as solid crystals, this protection of LD-fed OPN<sup>-/-</sup> mice from gallstone formation may be due to the remarkably lower biliary cholesterol concentration, with greater levels of bile acids and phospholipid and consequently a lower CSI.

Then we observed that the content of serum cholesterol in LD-fed OPN<sup>-/-</sup> mice was lower than that of LD-fed WT mice. And the fecal cholesterol output in LD-fed OPN<sup>-/-</sup> was increased in value, although the difference is not statistically significant. Additionally, the mRNA and protein expressions of NPC1L1, which is localized to the brush-border membrane of enterocytes (25) and plays an important role in intestinal cholesterol absorption (9), were both reduced in LD-fed

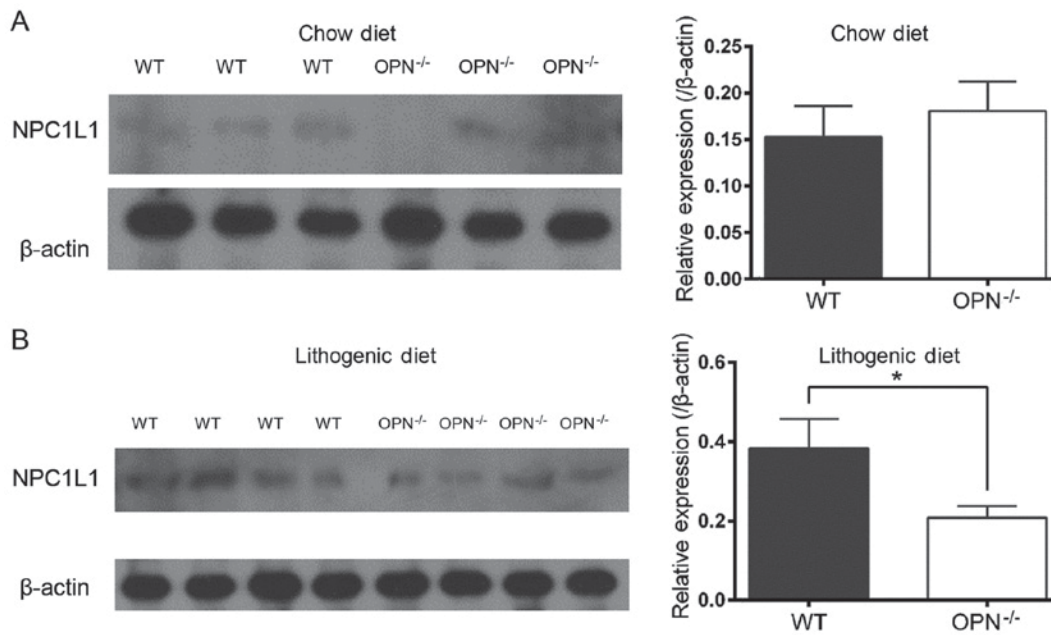


Figure 5. Western blot analysis of expression of protein NPC1L1 in mice. Ileum proteins were isolated from WT and  $OPN^{-/-}$  mice fed a (A) CD and (B) LD for 8 weeks, then analyzed by western blotting for NPC1L1 protein expression. The  $\beta$ -actin was selected as a control for gel loading. The relative average protein level was determined by densitometry. The data are expressed as the mean  $\pm$  SD. Statistical analysis was performed using unpaired Student's t test, \* $P < 0.05$ . WT, wild-type;  $OPN^{-/-}$ , OPN knockout; CD, chow diet; LD, lithogenic diet.

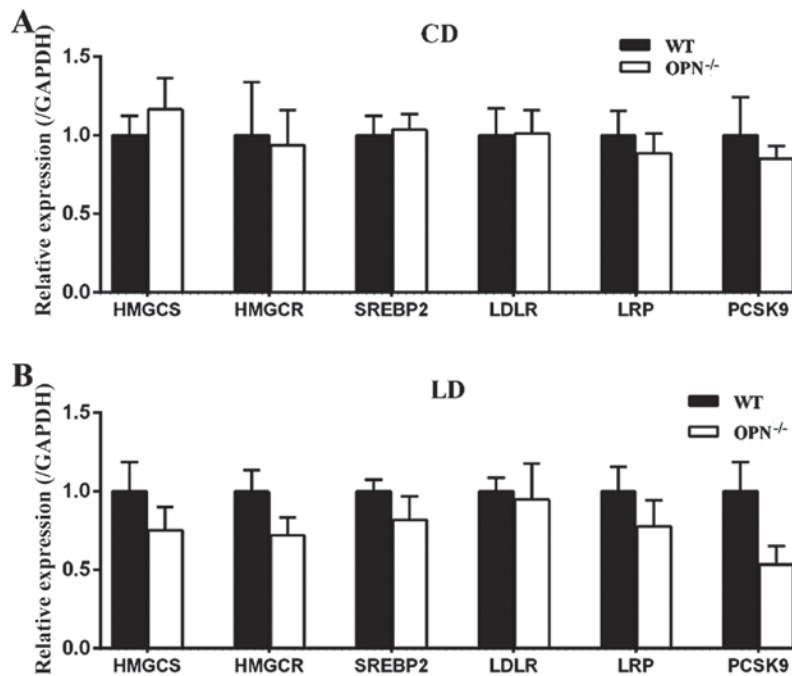


Figure 6. The mRNA expression of hepatic related genes in WT and  $OPN^{-/-}$  mice. Quantitative real-time PCR analysis of mRNA levels for hepatic related genes from WT mice and  $OPN^{-/-}$  mice fed a (A) CD and (B) LD for 8 weeks. The data are expressed as the mean  $\pm$  SD ( $n=6$  per group). Statistical analysis was performed by unpaired Student's t test. WT, wild-type;  $OPN^{-/-}$ , OPN knockout; CD, chow diet; LD, lithogenic diet.

$OPN^{-/-}$  mice. It is known that cholesterol derived from the intestine provides the first major source for cholesterol pool and influences biliary cholesterol secretion (26-28), and that high absorption of cholesterol enhances gallstone formation through this pathway (29). Therefore the participation of the NPC1L1 protein is very important, for it can determine the amount of cholesterol circulating to the liver and allow it to be excreted into bile. The increased level of intestinal NPC1L1

protein in gallbladder gallstone patients produces a high absorption of cholesterol (30). But inhibiting intestinal NPC1L1 expression reduces the intestinal absorption of cholesterol and prevents the formation of gallstone (31-34). Ezetimibe, which is a drug that blocks the expression of NPC1L1, could prevent cholesterol gallstone formation by effectively reducing intestinal cholesterol absorption and biliary cholesterol secretion (34,35). Although we did not measure the cholesterol

absorption rates during the mice experiments, these findings suggest that the protection of LD-fed OPN<sup>-/-</sup> mice from gallstone formation may be partially resulted from the reduced intestinal absorption of cholesterol, which is caused by the decreased expression of intestinal NPC1L1. However, it is a limitation that we have not performed NPC1L1 overexpression assays in mice to confirm the function of NPC1L1.

The expression of intestinal NPC1L1 is mainly regulated by LXR $\alpha$  (36) and PPAR $\delta$  (37). Unfortunately, we found no difference in the expression of LXR $\alpha$  nor PPAR $\delta$  between two mice strains. It suggests that the effect of OPN deficiency on intestinal NPC1L1 expression may not function through the LXR $\alpha$  or PPAR $\delta$  pathway. Further studies are necessary in order to fully address the mechanism by which OPN deficiency leads to intestinal NPC1L1 suppression.

For the increased biliary bile acids content in LD-fed OPN<sup>-/-</sup> mice, we did not find the expression of intestinal genes involved in reabsorption of bile acids differ between two strains. The reason might be that OPN could influence the expression of the cytochrome P450, family 7, subfamily a, polypeptide 1, the rate limiting enzyme of bile acids synthesis, as we found in our previous study (19).

In conclusion, we have described a previously unknown function of OPN, that it can affect intestinal absorption of cholesterol through NPC1L1, thus impacting the formation of gallstone in mice.

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