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Title Page

Title:

Hepatic expression of Niemann-Pick C1-Like 1, a cholesterol re-absorber from bile, exacerbates western diet-induced atherosclerosis in LDL receptor mutant mice.

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Running Title Page

Running title: Hepatic NPC1L1 exacerbates atherosclerosis

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Abbreviations:
ABCG5/G8, ATP-binding cassette transporter G5 and G8; CD, control diet; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; NPC1L1, Niemann-Pick C1-Like 1; VLDL, very low-density lipoprotein; WD, western diet; WT, wild-type.

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Abstract

Westernization of dietary habits increases lipid intake and is responsible for increased numbers of patients with atherosclerotic diseases. Niemann-Pick C1-Like 1 (NPC1L1)—a cholesterol importer—plays a crucial role in dietary cholesterol absorption in the intestine and is closely associated with several lipid-related diseases, including atherosclerosis. NPC1L1 is highly expressed in the liver and intestine in humans, whereas NPC1L1 expression is low in the rodent liver. Due to species differences in tissue distribution of NPC1L1, there are limited studies on the pathophysiological role of hepatic NPC1L1, a cholesterol re-absorber from bile. In the present study, to explore whether hepatic NPC1L1 is involved in development/progression of atherosclerosis, we compared four kinds of atherosclerosis mouse models with different expression levels of NPC1L1 in the intestinal and liver tissues in a genetic background of dysfunctional LDL receptor mutation. Western diet (WD)-induced hyperlipidemia and atherosclerotic plaque formation were more severe in mice expressing NPC1L1 in both the liver and intestine (plasma cholesterol: 839.5 mg/dL, plaque area: 29.5% of total aorta), compared with mice expressing NPC1L1 only in the intestine (plasma cholesterol: 573.1 mg/dL, plaque area: 13.3% of total aorta). Such hepatic NPC1L1-mediated promotion of hyperlipidemia and atherosclerosis was not observed in mice not expressing intestinal NPC1L1 and mice treated with ezetimibe, an NPC1L1 inhibitor used clinically for dyslipidemia. These results suggested that hepatic NPC1L1 promotes WD-induced dyslipidemia and atherosclerosis in concert with intestinal NPC1L1. Our findings provide novel insights into the pathophysiological importance of hepatic NPC1L1 in development/progression of atherosclerosis.

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Significance statement

NPC1L1, a cholesterol importer and a molecular target of ezetimibe clinically used for dyslipidemia, is highly expressed not only in the intestine, but also in the liver in humans, although pathophysiological importance of hepatic NPC1L1 in atherosclerotic diseases remained unclear. By using novel mouse models to separately analyze effects of hepatic and intestinal NPC1L1 on development/progression of atherosclerosis, we first demonstrated that hepatic NPC1L1 accelerates western diet-induced atherosclerotic plaque formation in an intestinal NPC1L1-dependent and an ezetimibe-sensitive manner.

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Introduction

Because of the global changes in dietary habits suiting western styles—such as high consumption of fats, including cholesterol—the number of patients with atherosclerosis—a chronic and progressive disease involving the arterial walls and resulting in lethal myocardial infarction and stroke—is increasing worldwide. It is well known that high plasma cholesterol concentration is one of the major risk factors for the development of atherosclerosis. Several processes such as *de novo* cholesterol synthesis, catabolism to bile acids, intestinal cholesterol absorption, and biliary cholesterol excretion, regulate plasma cholesterol levels. Among these processes, intestinal cholesterol absorption and biliary cholesterol excretion are controlled by Niemann-Pick C1-Like 1 (NPC1L1) protein (Altmann et al., 2004; Temel et al., 2007; Yamanashi et al., 2017; Yamanashi et al., 2007; Yamanashi et al., 2009; Yamanashi et al., 2018).

NPC1L1 is a cholesterol importer, which plays a key role in intestinal cholesterol absorption and is a molecular target of ezetimibe–a cholesterol absorption inhibitor clinically used for dyslipidemia (Altmann et al., 2004; Garcia-Calvo et al., 2005; Yamanashi et al., 2007). It has been reported that there are species differences in the tissue distribution of NPC1L1 (Altmann et al., 2004). Indeed, NPC1L1 is highly expressed in both the liver and intestine in humans, whereas NPC1L1 is predominantly expressed in the intestine but its expression is low in the liver of rodents. To date, the physiological functions of hepatic NPC1L1 have been studied extensively (Tang et al., 2011; Temel et al., 2007; Wang et al., 2018; Xie et al., 2013; Yamanashi et al., 2012) and several studies with liver-specific human NPC1L1 transgenic mice have demonstrated that hepatic NPC1L1 is expressed on the bile canalicular membrane, and is involved in re-absorption of biliary cholesterol by hepatocytes in an ezetimibe-sensitive manner (Tang et al., 2011; Temel et al., 2007; Wang et al., 2018; Xie et al., 2013). These findings suggest

that hepatic NPC1L1 negatively regulates biliary cholesterol excretion in humans.

To date, a number of *in vivo* studies with atherosclerosis mouse models, such as LDL receptor (LDLR)-deficient (or mutant) mice, and apolipoprotein E (apoE)-deficient mice, have been conducted to reveal molecular mechanisms of atherosclerosis development/progression and to identify novel therapeutic targets for atherosclerotic diseases (Davis et al., 2007; Knowles and Maeda, 2000; Lee et al., 2017; Zadelaar et al., 2007). Some of these studies indicated that NPC1L1-mediated intestinal cholesterol absorption is involved in atherosclerotic plaque formation and progression (Davis et al., 2007). However, due to differences in the tissue distribution of NPC1L1 between humans and rodents, a feasible pathophysiological involvement of hepatic NPC1L1 in atherosclerosis has been overlooked in many studies using mouse models. Indeed, although importance of hepatic NPC1L1 in (patho)physiology of bile formation and liver diseases has been intensively studied so far (Temel et al., 2007; Toyoda et al., 2019), there is little information about its effect on atherosclerosis progression. Therefore, the purpose of our study was to explore the effect of hepatic NPC1L1 on atherosclerotic plaque formation in mouse models.

For this purpose, we generated novel atherosclerosis mouse models by crossing LDLR mutant (*Ldlr*^{mt}) mice with liver-specific NPC1L1 transgenic (1L1^{Tg}) mice and/or NPC1L1 knockout (*1l1*^{KO}) mice, allowing us to independently analyze the effect of hepatic NPC1L1 and that of intestinal NPC1L1 on western diet (WD)-induced atherosclerosis development/progression. By comparing the generated four kinds of distinct mouse models with or without the expression of intestinal or hepatic NPC1L1 (*Ldlr*^{mt} mice, *Ldlr*^{mt}/1L1^{Tg} mice, *Ldlr*^{mt}/*1l1*^{KO} mice, and *Ldlr*^{mt}/1L1^{Tg}/*1l1*^{KO} mice), we revealed that hepatic NPC1L1 contributes to increased plasma concentrations of cholesterol and triglycerides and exacerbates

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atherosclerotic plaque formation when intestinal NPC1L1 is functional. These observations indicated the pathophysiological importance of NPC1L1 not only in the intestine, but also in the liver in diet-induced hyperlipidemia and atherosclerosis.

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Materials and methods

Animals

LDLR mutant (*Ldlr*^{mt}) mice with a C57BL/6 background (RBRC-GSC0247) were provided by RIKEN BRC through the National BioResource Project of MEXT (Yamamoto et al., 2017). *Npc1l1* knockout (*1l1*^{KO}) mice with a C57BL/6 background were previously generated (Takada et al., 2015). Liver-specific NPC1L1 transgenic (1L1^{Tg}) mice were purchased from The Jackson Laboratory and backcrossed with C57BL/6 mice. *Ldlr*^{mt} and *1l1*^{KO} mice were crossed to generate *Ldlr*-mutated *1l1*^{KO} mice (*Ldlr*^{mt}/*1l1*^{KO}). In addition, *Ldlr*-mutated 1L1^{Tg} mice (*Ldlr*^{mt}/1L1^{Tg}) were generated by crossing *Ldlr*^{mt} mice with 1L1^{Tg} mice. Furthermore, *Ldlr*^{mt}/*1l1*^{KO} mice and *Ldlr*^{mt}/1L1^{Tg} mice were crossed to generate *Ldlr*^{mt} mice expressing NPC1L1 in the liver, but not in the intestine (*Ldlr*^{mt}/1L1^{Tg}/*1l1*^{KO}). In the present study, the following four kinds of genetically distinct mice with or without NPC1L1 expression in the intestine or liver were tested: *Ldlr*^{mt} mice, *Ldlr*^{mt}/*1l1*^{KO} mice, *Ldlr*^{mt}/1L1^{Tg} mice, and *Ldlr*^{mt}/1L1^{Tg}/*1l1*^{KO} mice. All mice were housed in temperature- and humidity-controlled cages with a 12-h light-dark cycle and with free access to water and normal control diet (CD) [FR-1 (Funabashi Farm Co., Ltd., Chiba, Japan)] or WD [D12079B: 41kcal% fat and 0.21% cholesterol (Research Diets Inc., New Brunswick, NJ, USA)] in the absence or presence of ezetimibe (20 μg/g diet). Hepatic concentrations of ezetimibe (approximately 0.10 μg/g liver) and ezetimibe-glucuronide (approximately 1.0 μg/g liver), an active metabolite of ezetimibe, were not significantly different among mice of each genotype fed with the ezetimibe-containing WD. All experiments with mice were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with protocols approved by the Animal Studies Committee of the University of Tokyo. All mice studied were euthanized

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by cervical dislocation.

Western blot analysis

For preparing crude membranes from intestinal epithelial cells and hepatocytes, isolated mouse small intestine and liver were homogenized with buffer A (50 mM Tris-HCl (pH 7.4) containing 2 mM EDTA, 2 mM EGTA, 2 mM PMSF (phenylmethylsulfonyl fluoride), leupeptin (5 mg/mL), pepstatin (1 mg/mL), and oprotinin (5 mg/mL)) and centrifuged at 1,500 ×*g* for 15 min. After centrifugation, the supernatant was re-centrifuged at 20,000 ×*g* for 60 min. The precipitated crude membrane preparation was resuspended in buffer A and stored at -80°C before use in western blot analysis. The protein concentration of each specimen was measured by BCA assay using bovine serum albumin (BSA) as a standard. Total extracted crude membrane proteins were diluted with 2× SDS loading buffer and subjected to western blot analysis, as described previously (Yamanashi et al., 2007). An SDS-polyacrylamide gel (7%) was used to separate proteins in each specimen. The molecular weights were determined using a prestained protein marker (New England Biolabs Inc., Ipswich, MA, USA). The primary antibodies used for experiments were 500-fold diluted rabbit anti-NPC1L1 antibody (NB400-128) (Novus Biologicals, Centennial, CO, USA) for NPC1L1, or 200-fold diluted rabbit anti-Na^+/K^+-ATPase [Na^+- and K^+-dependent adenosine triphosphatase (ATPase)] α antibody [H-300 (sc-28800)] (Santa Cruz Biotechnology Inc., Dallas, TX, USA) for endogenous Na^+/K^+-ATPase α. For detection, the membrane was incubated in 5,000-fold diluted horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG) antibody (NA934V) (GE Healthcare UK Ltd., Buckinghamshire, UK) in TBS-T (Tris-buffered saline containing 0.05% Tween 20) with 0.1% BSA for 1 h at room temperature. Enzyme activity was assessed using an ECL Prime

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Western Blotting Detection Reagent (GE Healthcare) with a luminescent image analyzer (Bio-Rad Laboratories, Hercules, CA, USA).

Emulsion preparation

Emulsions were prepared as described previously (Narushima et al., 2008; Takada et al., 2015; Yamamoto et al., 2007) with minor modifications. Briefly, stock lipid solutions were mixed to give a final concentration of 13.3 mM triolein, 2.6 mM cholesterol, 3 mM L-α-phosphatidylcholine, and 3 μCi/mL [^{3}H]cholesterol (American Radiolabeled Chemical Inc., Saint Louis, MO, USA). The solvent was evaporated, and 19 mM sodium taurocholate [dissolved in phosphate buffered saline (PBS)] was added to give the required lipid concentrations. The mixture was then sonicated three times for 3 min using an ultrasonic homogenizer [UP 200H (Hielscher Ultrasonics, Teltow, Germany)].

***In vivo* acute cholesterol absorption study**

In vivo acute cholesterol absorption studies were conducted as described previously (Narushima et al., 2008; Takada et al., 2015; Yamamoto et al., 2007) with minor modifications. Ten- to twelve-week-old male mice were fasted for 18 h and anesthetized with urethane. Next, a [^{3}H]cholesterol-containing emulsion (5 mL/kg) was delivered directly into the small intestine via the duodenal cannula. Two hours after the emulsion loading, mice were sacrificed, and plasma and liver were isolated to quantify the level of [^{3}H]cholesterol. The level of [^{3}H]cholesterol in each specimen was measured using the liquid scintillation counter.

Quantification of biliary lipids

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Ten- to twelve-week-old male mice were anesthetized with urethane. Then, the cystic duct was ligated and a common bile duct fistula was created using a Teflon catheter [UT-03 (Unique Medical Co., Ltd., Tokyo, Japan)] to collect hepatic bile specimens as described previously (Yamanashi et al., 2011). Concentrations of biliary cholesterol, total bile acids, and phospholipids were quantified using Total Cholesterol E-test Wako (Wako, Osaka, Japan), Total bile acids test Wako (Wako), and Phospholipids C-test Wako (Wako), respectively.

Lipoprotein fractionation

Lipoprotein fractionation was performed as described previously (Yamamoto et al., 2017). Briefly, plasma specimens collected from mice were fractionated by size-exclusion chromatography using a Pharmacia Smart System fast protein liquid chromatography (FPLC) system equipped with a Superose 6 column (GE Healthcare). Elution was performed in PBS containing 1 mM EDTA and 3 mM sodium azide as a running buffer. After loading 300 μL of serum, the system was run with a constant flow of 200 μL/min, with 500 μL fractions collected. Cholesterol and triglyceride concentrations in each fraction were quantified with the Total Cholesterol E-test Wako and Triglyceride E-test Wako (Wako), respectively.

Quantification of hepatic lipids

Lipid extraction from liver samples was performed according to the well-known Bligh and Dyer method (Bligh and Dyer, 1959). In brief, 200 μL of homogenized liver solution (50 mg of liver/mL of PBS) was mixed with 250 μL of chloroform and 500 μL of methanol, then well vortexed. After 10 min, 250 μL of chloroform was added to the mixture, and after blending, the homogenates mixture was centrifuged at 1,800 ×*g* for 10 min. After complete removal of

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the alcoholic (top) layer, 250 μL of the resulting chloroform (bottom) layer was transferred to a new glass tube, then evaporated to dryness under a stream of nitrogen, dissolved in isopropanol containing 10% (w/w) Triton X-100, and subjected to measurements of hepatic cholesterol and triglyceride. For quantitative calibration curves, standard samples containing known concentrations of cholesterol and triglyceride were prepared in a similar manner. The concentrations of cholesterol and triglyceride in each sample were measured using Total Cholesterol E-test Wako and Triglyceride E-test Wako, respectively.

RNA extraction and quantitative PCR

Total RNA was extracted from mouse liver using the RNA isoPlus Reagent (Takara Bio, Inc, Shiga, Japan), according to the manufacturer's protocol. The extracted total RNA was reverse-transcribed with ReverTraAce (Toyobo, Osaka, Japan). Quantitative PCR was performed using SYBR GreenER qPCR SuperMix Universal (Life Technologies, Tokyo Japan) and Eco Real-Time PCR System (Illumina Inc., San Diego, CA, USA) at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Primers for mouse 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase (sense and antisense primers were 5′-gtggcaatgctgatcgg-3′ and 5′-ggatcttcttgcggtaggct-3′, respectively), mouse HMG-CoA reductase (sense and antisense primers were 5′-tgggcatgaacatgatctcta-3′ and 5′-ggcttcacaaaccacagtc-3′, respectively), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense and antisense primers were 5′-atgtgtccgtcgtggatctg-3′ and 5′-tgaagtcgcaggagacaacc-3′, respectively) were used.

Atherosclerotic lesion analysis

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Six- to eight-week-old male mice were fed with CD or WD for 20 weeks. After the 20-weeks of feeding, mice were sacrificed followed by plasma collection, and the entire aorta was isolated. The collected aorta was opened longitudinally, and stained with Oil Red O (Merck KGaA, Darmstadt, Germany). Oil Red O-stained plaque lesions were quantified using Adobe Photoshop, and the extent of atherosclerosis was expressed as the percentage of the lesion area extending from the ascending aorta to the abdominal bifurcation. The quantification was performed by an investigator who was blinded to group allocation.

Sample sizes

Each experiment was designed to use the minimum number of mice to obtain informative results. Although no statistical methods were used to pre-determine sample sizes, based on preliminary results or an empirical approach we determined sufficient sample size. In assays for Figures 3 and 5 requiring a long-term western diet-feeding, we used all mice born for the assays. Because birth rates of mice of each genotype were different, sample sizes of these assays differed among genotypes.

Data analysis and statistics

Individual data points are shown as dot plots and graph bars represent means ± SD. All statistical analyses were performed by using GraphPad PRISM 8 (GraphPad Software, San Diego, CA, USA). Different statistical tests were used for different experiments as described in the figure legends. Briefly, we pre-determined to statistically compare *Ldlr*^{mt}/*111*^{KO} mice, *Ldlr*^{mt}/1L1^{Tg} mice, and *Ldlr*^{mt}/1L1^{Tg}/*111*^{KO} mice to *Ldlr*^{mt} mice under conditions without ezetimibe administration by Dunnett's test. In addition, we pre-determined to statistically

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compare ezetimibe-administered mice and non-administered mice in each genotype by Student's *t*-test. Statistical significance was defined in terms of P values less than 0.05.

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Results

Generated mouse models showed expected NPC1L1 protein expression patterns in the intestine and liver.

First, we examined NPC1L1 protein expression in the liver and intestine in our generated mouse models (Fig. 1). Consistent with previous observations in wild-type mice (Altmann et al., 2004), *Ldlr*^{mt} mice exhibited distinct expression of NPC1L1 in the intestine, whereas hepatic NPC1L1 was hardly detected. Meanwhile, both intestinal and hepatic expression of NPC1L1 protein was observed in *Ldlr*^{mt}/1L1^{Tg} mice, whereas neither intestinal nor hepatic NPC1L1 was detected in *Ldlr*^{mt}/*1l1*^{KO} mice. In addition, we confirmed expression of NPC1L1 in the liver, but little in the intestine, in *Ldlr*^{mt}/1L1^{Tg}/*1l1*^{KO} mice. Based on the tissue distribution of NPC1L1, we have described *Ldlr*^{mt}, *Ldlr*^{mt}/1L1^{Tg}, *Ldlr*^{mt}/*1l1*^{KO}, and *Ldlr*^{mt}/1L1^{Tg}/*1l1*^{KO} mice as I^+L^-, I^+L^+, I^-L^-, and I^-L^+ mice (I: intestine, L: liver), respectively, in the following text.

Generated mouse models have appropriate activities of intestinal cholesterol absorption and biliary cholesterol excretion.

We next examined intestinal cholesterol absorption in mice of each genotype (Fig. 2A). Consistent with the expression level of NPC1L1 protein in the intestine, cholesterol absorption into the plasma and the liver after oral administration was lower in I^-L^- and I^-L^+ mice, compared with that in I^+L^- mice (wild-type mice for tissue distribution of NPC1L1 (WT_{1L1})). Meanwhile, little difference was observed between I^+L^- (WT_{1L1}) mice and I^+L^+ mice.

We also elucidated biliary cholesterol excretion in the mice of each genotype (Fig. 2B). While biliary cholesterol concentration in I^-L^- mice was equivalent to that in I^+L^- (WT_{1L1}) mice, I^-L^+ and I^+L^+ mice exhibited a lower concentration of biliary cholesterol than I^+L^- (WT_{1L1})

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mice. Next, we confirmed that the concentration of other biliary components, such as bile acids and phospholipids, was similar among all groups. These observations indicated that, consistent with previous reports (Temel et al., 2007), hepatic NPC1L1 selectively re-absorbs biliary cholesterol in the liver.

All of these data indicated that we successfully generated mouse models with different expression patterns of NPC1L1, and varied cholesterol (re-)uptake activity in the intestine and liver.

Hepatic NPC1L1 exacerbated WD-induced dyslipidemia.

Next, we analyzed plasma lipid levels and lipoprotein profiles in the mice of each genotype after WD-feeding for 20 weeks, with or without ezetimibe. We observed that body weights of all mice increased appropriately during the assay period, indicating that mice of each genotype grew normally under our experimental conditions (Supplemental Table 1). In addition, before WD-feeding, we did not observe a significant difference in the plasma levels of cholesterol (Supplemental Fig. 1A) and triglycerides (Supplemental Fig. 1B) among the four genotypes. Then, we analyzed the effect of WD-feeding on plasma lipid levels and found that, in I^+L^- (WT_{1L1}) mice and I^+L^+ mice, plasma concentrations of cholesterol and triglyceride were dramatically increased and that these increases were effectively inhibited upon ezetimibe treatment (Fig. 3A-B). Meanwhile, in intestinal NPC1L1-deficient mice (I^-L^- and I^-L^+ mice), an ezetimibe-sensitive increase in plasma lipids was hardly observed, and consequently, plasma lipid levels in these mice were significantly lower than those in mice expressing NPC1L1 in the intestine (I^+L^- (WT_{1L1}) and I^+L^+ mice). These results indicated that intestinal NPC1L1 plays a critical role in the diet-induced increase in plasma lipid levels. Moreover, we observed that

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I^+L^+ mice exhibited significantly higher levels of plasma lipids than I^+L^- (WT_{1L1}) mice, whereas little difference in plasma lipid levels was observed between I^-L^- and I^-L^+ mice (Fig. 3A-B). These results suggested that hepatic NPC1L1 also contributes to increase in plasma lipids when intestinal NPC1L1 is functional.

We also analyzed lipoprotein profiles in the mice of each genotype after WD-feeding and found that the ezetimibe-sensitive increases in plasma cholesterol and triglyceride were primarily accounted for by ezetimibe-sensitive increases in VLDL and/or LDL (VLDL/LDL)-cholesterol (Fig. 3C), and VLDL/LDL-triglyceride (Fig. 3D), respectively. These findings indicated that both hepatic and intestinal NPC1L1 are somehow associated with increased levels of VLDL/LDL-lipids in particular.

Since the liver is a major source of plasma lipids, we analyzed hepatic concentrations of cholesterol and triglyceride in the mice of each genotype after WD-feeding for 20 weeks. An ezetimibe-sensitive WD-induced hepatic cholesterol accumulation was significantly increased in I^+L^+ mice compared with that in I^+L^- (WT_{1L1}) mice, and such an accumulation was not observed in intestinal NPC1L1-deficient mice (I^-L^- and I^-L^+ mice) (Fig. 4A). Similar tendency was observed in triglyceride levels in the liver, although the difference between I^+L^- (WT_{1L1}) and I^+L^+ mice was not statistically significant (Fig. 4B). We also analyzed hepatic mRNA expressions of genes involved in *de novo* cholesterol synthesis (HMG-CoA synthase and HMG-CoA reductase) and found that no significant difference was observed between I^+L^- (WT_{1L1}) and I^+L^+ mice, whereas the expression levels of HMG-CoA reductase in I^-L^- and I^-L^+ mice were significantly higher than that in I^+L^- (WT_{1L1}) mice (Supplemental Fig. 2). These results, together with the fact that hepatic NPC1L1 is involved in biliary cholesterol re-absorption (Fig. 2B), suggested that higher levels of plasma and hepatic cholesterol in I^+L^+ mice compared to

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I^+L^- (WT_{1L1}) mice (Figs. 3A and 4A, respectively) were probably not due to an enhancement of *de novo* cholesterol synthesis in the liver, but rather to the reduced biliary cholesterol excretion.

Hepatic NPC1L1 exacerbated WD-induced atherosclerotic plaque formation.

Next, we analyzed progression of WD-induced atherosclerosis in the mice of each genotype (Fig. 5A). As a result, atherosclerotic plaques, which were stained by Oil Red O, were observed in I^+L^- (WT_{1L1}) mice after WD-feeding for 20 weeks. Notably, I^+L^+ mice developed atherosclerotic plaques more severely than I^+L^- (WT_{1L1}) mice. In addition, we observed that mice that did not express intestinal NPC1L1 (I^-L^- mice and I^-L^+ mice) hardly developed atherosclerotic plaques after WD-feeding. These results were in accordance with plasma lipid levels. Indeed, we observed significant positive correlations of atherosclerotic plaque area with plasma cholesterol levels (Fig. 5B) and triglyceride levels (Fig. 5C). In particular, plasma cholesterol levels exhibited a strong correlation with the plaque area. These observations indicated that, although intestinal NPC1L1 clearly contributes to atherosclerotic plaque formation as well as hyperlipidemia progression, hepatic NPC1L1 can also exacerbate diet-induced hyperlipidemia and atherosclerotic plaque formation/progression in concert with intestinal NPC1L1 (Fig. 6). Furthermore, we also found that ezetimibe administration inhibited plaque formation in not only I^+L^- (WT_{1L1}) mice, but also in I^+L^+ mice (Fig. 5). These results, together with previous findings suggesting that ezetimibe can inhibit both intestinal and hepatic NPC1L1 *in vivo* (Temel et al., 2007), provide a strong rationale for using ezetimibe to prevent atherosclerosis in humans, who express NPC1L1 in both the intestine and liver.

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Discussion

In this study, we generated mouse models to analyze the effects of NPC1L1 in the intestine and liver on atherosclerosis development/progression (Figs. 1 and 2), and demonstrated that, under a WD-fed condition, mice expressing NPC1L1 in both the intestine and liver (I^+L^+ mice) showed greater increases in serum VLDL/LDL lipids (Fig. 3) and hepatic lipids (Fig. 4), and more severe atherosclerotic plaque formation (Fig. 5) than mice expressing NPC1L1 predominantly in the intestine and to a lesser extent in the liver (I^+L^- (WT_{1L1}) mice). In addition, consistent with a previous study using apoE-deficient mouse models (Davis et al., 2007), NPC1L1 knockout mice (I^-L^- mice) hardly exhibited WD-induced increases in serum lipids (Fig. 3) and atherosclerotic plaques (Fig. 5). Taken together, our observations suggest that hepatic NPC1L1 can exacerbate diet-induced dyslipidemia and atherosclerosis, in concert with intestinal NPC1L1 (Fig. 6). Considering that NPC1L1 is highly expressed in the liver, in addition to the intestine in humans (Altmann et al., 2004), I^+L^+ mice will be useful as an animal model for deeper understandings of the pathophysiological function (involvement) of not only intestinal, but also hepatic NPC1L1, in atherosclerotic diseases.

WD-fed mice showed an increase in VLDL/LDL-lipids in an intestinal NPC1L1-dependent manner, whereas HDL lipids were hardly affected by NPC1L1 expression (Fig. 3C-D). This is consistent with a previous report demonstrating that intestinal NPC1L1 is a major determinant of plasma VLDL/LDL-cholesterol levels in LDLR-deficient mice (Xie et al., 2014). In addition, we revealed that, besides intestinal NPC1L1, hepatic NPC1L1 also contributes to increased VLDL/LDL-lipids (Fig. 3C-D), indicating that NPC1L1-mediated re-absorption of biliary cholesterol is involved in the regulation of VLDL/LDL-lipid levels. In accordance with all of these observations, a clinical study demonstrated that plasma LDL-cholesterol levels were

significantly lower in carriers of NPC1L1 inactivating mutations than those in non-carriers of such mutations, whereas HDL-cholesterol levels were not significantly different between carriers and non-carriers (Myocardial Infarction Genetics Consortium et al., 2014). The detailed mechanisms involving how NPC1L1 controls concentrations of VLDL/LDL-lipids remains unknown. However, considering our observations that hepatic NPC1L1 increased hepatic cholesterol level when intestinal NPC1L1 is functional (Fig. 4A), and a previous finding that cholesterol (ester) in the liver stimulates hepatic production and/or secretion of VLDL into the blood (Fungwe et al., 1992), in addition to the fact that VLDL is converted to LDL in the bloodstream, it is highly possible that NPC1L1 indirectly regulates blood VLDL/LDL levels by controlling hepatic cholesterol levels through dietary cholesterol absorption and biliary cholesterol re-absorption. Previous findings that hepatic overexpression of a heterodimer of ATP-binding cassette transporter G5 and G8 (ABCG5/G8), a cholesterol exporter from hepatocytes to the bile (Berge et al., 2000; Yu et al., 2002), reduced blood VLDL/LDL levels, and inhibited atherosclerosis progression in LDLR-deficient mice (Basso et al., 2007) also support the potential importance of hepatic cholesterol homeostasis regulated by biliary cholesterol excretion via the cholesterol transporter-mediated pathway.

We observed that ezetimibe treatment could improve dyslipidemia and effectively inhibited diet-induced atherosclerotic plaque formation in I^+L^+ mice and I^+L^- (WT_{1L1}) mice (Figs. 3 and 5). Consistent with our results using mouse models, a number of clinical researches demonstrated that ezetimibe therapy was effective to improve dyslipidemia in humans (Bays, 2002). However, it seems that the extent of therapeutic (or preventive) effects of ezetimibe on atherosclerotic diseases are not as high in humans as those observed in I^+L^+ mice in this study. Indeed, several large-scale clinical trials demonstrated that positive effects of ezetimibe on

atherosclerotic diseases in humans were not observed (intima-media thickness was not improved by ezetimibe administration (Kastelein et al., 2008; Taylor et al., 2009)) or were very limited (only 2% reduction in the rate of cardiovascular events with ezetimibe therapy (Cannon et al., 2015)). The reason why such a difference exits has not yet been clarified, but the timing of starting ezetimibe treatment may be a key factor. In our study as well as many other studies with mouse models, ezetimibe treatment was initiated at the same timing as starting WD-feeding, and therefore, the tested mice had not experienced severe dyslipidemia. Meanwhile, most of the clinical studies examining the effect of ezetimibe were conducted with patients already diagnosed with dyslipidemia. These facts, together with results of meta-analyses showing that LDL-cholesterol has a cumulative effect on the risk of cardiovascular diseases (Horton et al., 2009), suggest that the difference in timing of starting ezetimibe therapy may account for the difference in therapeutic effects of ezetimibe on atherosclerotic diseases. In support of this, it has been revealed that humans with dysfunctional mutations of the *NPC1L1* gene exhibited significant decreases in the risk of cardiovascular diseases, compared to non-carriers of such mutations (Myocardial Infarction Genetics Consortium et al., 2014), indicating that lifelong reduction in dietary and biliary cholesterol (re-)absorption can also prevent cardiovascular diseases in humans. From all of these results, starting ezetimibe therapy and/or improvement of dietary habits at the earliest possible stage of dyslipidemia could be important in preventing development/progression of atherosclerotic diseases.

In conclusion, we revealed that hepatic NPC1L1 exacerbates diet-induced dyslipidemia and atherosclerosis when intestinal NPC1L1 is functional (Fig. 6). To our knowledge, this is the first report demonstrating that hepatic NPC1L1 affects diet-induced atherosclerosis development/progression. Unfortunately, the detailed mechanisms involved have not been

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clarified. We recently revealed that NPC1L1 has uptake activity not only for cholesterol, but also for vitamins E and K_1 (Narushima et al., 2008; Takada and Suzuki, 2010; Takada et al., 2015; Yamanashi et al., 2017). As the chemical structures of cholesterol, vitamin E, and vitamin K_1, are largely different (Yamanashi et al., 2017), it is possible that NPC1L1 might exhibit unexpectedly broad substrate specificity and might be involved in the (re-)absorption of various dietary and/or biliary lipids, including unknown harmful lipids causing and/or promoting atherosclerosis. Considering that vitamins E and K_1 demonstrate positive effects on inhibiting atherosclerosis (vitamin E: antioxidaive effect, vitamin K_1: anticalcification effect), the balance of harmful lipids and beneficial lipids (e.g. vitamin E and vitamin K_1) controlled by NPC1L1 should be taken into consideration for understanding atherosclerosis development/progression. From this point of view, comprehensive analyses of plasma, hepatic, and biliary lipids in our generated mouse models will be useful for deeper understanding of the NPC1L1-mediated exacerbation of atherosclerosis.

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Author contributions

Participated in research design: Yamamoto, Yamanashi, Takada, Suzuki

Conducted experiments: Yamamoto, Yamanashi, Mu, Tanaka, Komine

Performed data analysis: Yamamoto, Yamanashi, Takada

Wrote or contributed to the writing of the manuscript: Yamanashi, Takada, Suzuki

References

Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N and Graziano MP (2004) Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* **303**(5661): 1201-1204.

Basso F, Freeman LA, Ko C, Joyce C, Amar MJ, Shamburek RD, Tansey T, Thomas F, Wu J, Paigen B, Remaley AT, Santamarina-Fojo S and Brewer HB, Jr. (2007) Hepatic ABCG5/G8 overexpression reduces apoB-lipoproteins and atherosclerosis when cholesterol absorption is inhibited. *Journal of lipid research* **48**(1): 114-126.

Bays H (2002) Ezetimibe. *Expert opinion on investigational drugs* **11**(11): 1587-1604.

Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R and Hobbs HH (2000) Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* **290**(5497): 1771-1775.

Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**(8): 911-917.

Cannon CP, Blazing MA, Giugliano RP, McCagg A, White JA, Theroux P, Darius H, Lewis BS, Ophuis TO, Jukema JW, De Ferrari GM, Ruzyllo W, De Lucca P, Im K, Bohula EA, Reist C, Wiviott SD, Tershakovec AM, Musliner TA, Braunwald E, Califf RM and Investigators I-I (2015) Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. *The New England journal of medicine* **372**(25): 2387-2397.

Davis HR, Jr., Hoos LM, Tetzloff G, Maguire M, Zhu LJ, Graziano MP and Altmann SW (2007) Deficiency of Niemann-Pick C1 Like 1 prevents atherosclerosis in ApoE-/- mice. *Arteriosclerosis, thrombosis, and vascular biology* **27**(4): 841-849.

Fungwe TV, Cagen L, Wilcox HG and Heimberg M (1992) Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol. *Journal of lipid research* **33**(2): 179-191.

Garcia-Calvo M, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, Crona JH, Davis HR, Jr., Dean DC, Detmers PA, Graziano MP, Hughes M, Macintyre DE, Ogawa A, O'Neill K A, Iyer SP, Shevell DE, Smith MM, Tang

YS, Makarewicz AM, Ujjainwalla F, Altmann SW, Chapman KT and Thornberry NA (2005) The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proceedings of the National Academy of Sciences of the United States of America* **102**(23): 8132-8137.

Horton JD, Cohen JC and Hobbs HH (2009) PCSK9: a convertase that coordinates LDL catabolism. *Journal of lipid research* **50 Suppl**: S172-177.

Kastelein JJ, Akdim F, Stroes ES, Zwinderman AH, Bots ML, Stalenhoef AF, Visseren FL, Sijbrands EJ, Trip MD, Stein EA, Gaudet D, Duivenvoorden R, Veltri EP, Marais AD, de Groot E and Investigators E (2008) Simvastatin with or without ezetimibe in familial hypercholesterolemia. *The New England journal of medicine* **358**(14): 1431-1443.

Knowles JW and Maeda N (2000) Genetic modifiers of atherosclerosis in mice. *Arteriosclerosis, thrombosis, and vascular biology* **20**(11): 2336-2345.

Lee YT, Lin HY, Chan YW, Li KH, To OT, Yan BP, Liu T, Li G, Wong WT, Keung W and Tse G (2017) Mouse models of atherosclerosis: a historical perspective and recent advances. *Lipids in health and disease* **16**(1): 12.

Myocardial Infarction Genetics Consortium I, Stitziel NO, Won HH, Morrison AC, Peloso GM, Do R, Lange LA, Fontanillas P, Gupta N, Duga S, Goel A, Farrall M, Saleheen D, Ferrario P, Konig I, Asselta R, Merlini PA, Marziliano N, Notarangelo MF, Schick U, Auer P, Assimes TL, Reilly M, Wilensky R, Rader DJ, Hovingh GK, Meitinger T, Kessler T, Kastrati A, Laugwitz KL, Siscovick D, Rotter JI, Hazen SL, Tracy R, Cresci S, Spertus J, Jackson R, Schwartz SM, Natarajan P, Crosby J, Muzny D, Ballantyne C, Rich SS, O'Donnell CJ, Abecasis G, Sunaev S, Nickerson DA, Buring JE, Ridker PM, Chasman DI, Austin E, Kullo IJ, Weeke PE, Shaffer CM, Bastarache LA, Denny JC, Roden DM, Palmer C, Deloukas P, Lin DY, Tang ZZ, Erdmann J, Schunkert H, Danesh J, Marrugat J, Elosua R, Ardissino D, McPherson R, Watkins H, Reiner AP, Wilson JG, Altshuler D, Gibbs RA, Lander ES, Boerwinkle E, Gabriel S and Kathiresan S (2014) Inactivating mutations in NPC1L1 and protection from coronary heart disease. *The New England journal of medicine* **371**(22): 2072-2082.

Narushima K, Takada T, Yamanashi Y and Suzuki H (2008) Niemann-pick C1-like 1 mediates alpha-tocopherol transport. *Molecular pharmacology* **74**(1): 42-49.

Takada T and Suzuki H (2010) Molecular mechanisms of membrane transport of

vitamin E. *Molecular nutrition & food research* **54**(5): 616-622.

Takada T, Yamanashi Y, Konishi K, Yamamoto T, Toyoda Y, Masuo Y, Yamamoto H and Suzuki H (2015) NPC1L1 is a key regulator of intestinal vitamin K absorption and a modulator of warfarin therapy. *Science translational medicine* **7**(275): 275ra223.

Tang W, Jia L, Ma Y, Xie P, Haywood J, Dawson PA, Li J and Yu L (2011) Ezetimibe restores biliary cholesterol excretion in mice expressing Niemann-Pick C1-Like 1 only in liver. *Biochimica et biophysica acta* **1811**(9): 549-555.

Taylor AJ, Villines TC, Stanek EJ, Devine PJ, Griffen L, Miller M, Weissman NJ and Turco M (2009) Extended-release niacin or ezetimibe and carotid intima-media thickness. *The New England journal of medicine* **361**(22): 2113-2122.

Temel RE, Tang W, Ma Y, Rudel LL, Willingham MC, Ioannou YA, Davies JP, Nilsson LM and Yu L (2007) Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *The Journal of clinical investigation* **117**(7): 1968-1978.

Toyoda Y, Takada T, Umezawa M, Tomura F, Yamanashi Y, Takeda K and Suzuki H Identification of hepatic NPC1L1 as an NAFLD risk factor evidenced by ezetimibe-mediated steatosis prevention and recovery. *FASEB BioAdvances* **1**(5): 283-295.

Wang Y, Tang W, Yang P, Shin H and Li Q (2018) Hepatic NPC1L1 promotes hyperlipidemia in LDL receptor deficient mice. *Biochemical and biophysical research communications* **499**(3): 626-633.

Xie P, Jia L, Ma Y, Ou J, Miao H, Wang N, Guo F, Yazdanyar A, Jiang XC and Yu L (2013) Ezetimibe inhibits hepatic Niemann-Pick C1-Like 1 to facilitate macrophage reverse cholesterol transport in mice. *Arteriosclerosis, thrombosis, and vascular biology* **33**(5): 920-925.

Xie P, Zhu H, Jia L, Ma Y, Tang W, Wang Y, Xue B, Shi H and Yu L (2014) Genetic demonstration of intestinal NPC1L1 as a major determinant of hepatic cholesterol and blood atherogenic lipoprotein levels. *Atherosclerosis* **237**(2): 609-617.

Yamamoto H, Takada T, Yamanashi Y, Ogura M, Masuo Y, Harada-Shiba M and Suzuki H (2017) VLDL/LDL acts as a drug carrier and regulates the transport and metabolism of drugs in the body. *Scientific reports* **7**(1): 633.

Yamamoto T, Ito K, Honma M, Takada T and Suzuki H (2007) Cholesterol-lowering

effect of ezetimibe in uridine diphosphate glucuronosyltransferase 1A-deficient (Gunn) rats. *Drug metabolism and disposition: the biological fate of chemicals* **35**(9): 1455-1458.

Yamanashi Y, Takada T, Kurauchi R, Tanaka Y, Komine T and Suzuki H (2017) Transporters for the Intestinal Absorption of Cholesterol, Vitamin E, and Vitamin K. *Journal of atherosclerosis and thrombosis* **24**(4): 347-359.

Yamanashi Y, Takada T, Shoda J and Suzuki H (2012) Novel function of Niemann-Pick C1-like 1 as a negative regulator of Niemann-Pick C2 protein. *Hepatology* **55**(3): 953-964.

Yamanashi Y, Takada T and Suzuki H (2007) Niemann-Pick C1-like 1 overexpression facilitates ezetimibe-sensitive cholesterol and beta-sitosterol uptake in CaCo-2 cells. *The Journal of pharmacology and experimental therapeutics* **320**(2): 559-564.

Yamanashi Y, Takada T and Suzuki H (2009) In-vitro characterization of the six clustered variants of NPC1L1 observed in cholesterol low absorbers. *Pharmacogenetics and genomics* **19**(11): 884-892.

Yamanashi Y, Takada T and Suzuki H (2018) Associations between Lifestyle-Related Diseases and Transporters Involved in Intestinal Absorption and Biliary Excretion of Cholesterol. *Biological & pharmaceutical bulletin* **41**(1): 1-10.

Yamanashi Y, Takada T, Yoshikado T, Shoda J and Suzuki H (2011) NPC2 regulates biliary cholesterol secretion via stimulation of ABCG5/G8-mediated cholesterol transport. *Gastroenterology* **140**(5): 1664-1674.

Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC and Hobbs HH (2002) Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *The Journal of clinical investigation* **110**(5): 671-680.

Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM and Kooistra T (2007) Mouse models for atherosclerosis and pharmaceutical modifiers. *Arteriosclerosis, thrombosis, and vascular biology* **27**(8): 1706-1721.

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Footnotes

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H.Y., Y.Y., T.T., and H.S. have a patent pending related to the work reported in this paper.

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Legends for Figures

Figure 1. NPC1L1 protein expression in *NPC1L1* gene-modified LDLR mutant mice. Protein expression of NPC1L1 in the intestine and liver from 10-12-week-old male mice was analyzed by western blot analysis. Na^+/K^+-ATPase α was used as loading control.

Figure 2. Characterization of *NPC1L1* gene-modified LDLR mutant mice. (A) Cholesterol absorption was examined in 10-12-week-old male I^+L^-, I^+L^+, I^-L^-, and I^-L^+ mice. [^{3}H]cholesterol concentrations in plasma, liver, and plasma + liver were examined 2 h after the administration of [^{3}H]cholesterol-containing emulsion. **(B)** Biliary concentrations of cholesterol, bile acids, and phospholipids were examined in 10-12-week-old male I^+L^-, I^+L^+, I^-L^-, and I^-L^+ mice. Bar graphs represent means ± SD. The dots on bar graphs represent each individual data point (n=3: No statistical analysis was performed in Figure 2 because the sample size was too small to justify statistical testing for multiple comparison, although observed differences among genotypes were sufficiently clear to validate the constructed mouse models.).

Figure 3. Plasma lipid profiles in *NPC1L1* gene-modified LDLR mutant mice after western diet feeding. (A) Cholesterol and **(B)** triglyceride concentrations in plasma from male mice (initially 6-8-week-old) fed with a western diet (WD) for 20 weeks in the absence (−) or presence (+) of ezetimibe (Eze). Bar graphs represent means ± SD. The dots on bar graphs represent each individual data point [n=15: I^+L^- Eze (−) mice, n=8: I^+L^- Eze (+) mice, n=9: I^+L^+ Eze (−) mice, n=4: I^+L^+ Eze (+) mice, n=12: I^-L^- Eze (−) mice, n=4: I^-L^- Eze (+) mice, n=7: I^-L^+ Eze (−) mice, n=4: I^-L^+ Eze (+) mice]. $^*p<0.05$ and $^{**}p<0.01$, significantly different by Dunnett's test comparing I^+L^+ Eze (−) mice, I^-L^- Eze (−) mice, and I^-L^+ Eze (−) mice with

I^+L^- Eze (−) mice. $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$, significantly different by Student's *t*-test comparing two groups [Eze (−) and Eze (+)] in each genotype. N.S., not significantly different between the groups. **(C)** Cholesterol and **(D)** triglyceride concentrations in lipoprotein fractions collected from male mice (initially 6-8-week-old) fed with a WD for 20 weeks in the absence (left panel) or presence (right panel) of ezetimibe. Representative lipoprotein profiles from at least two independent experiments are shown.

Figure 4. Hepatic lipid concentrations in *NPC1L1* gene-modified LDLR mutant mice after western diet feeding. (A) Cholesterol and **(B)** triglyceride concentrations in the liver from male mice (initially 6-8-week-old) fed with a western diet (WD) for 20 weeks in the absence (−) or presence (+) of ezetimibe (Eze). Bar graphs represent means ± SD. The dots on bar graphs represent each individual data point (n=4). $^{**}p<0.01$, significantly different by Dunnett's test comparing I^+L^+ Eze (−) mice, I^-L^- Eze (−) mice, and I^-L^+ Eze (−) mice with I^+L^- Eze (−) mice. $^{\dagger\dagger}p<0.01$, significantly different by Student's *t*-test comparing two groups [Eze (−) and Eze (+)] in each genotype. N.S., not significantly different between the groups.

Figure 5. Atherosclerotic lesions of the aorta in *NPC1L1* gene-modified LDLR mutant mice after western diet feeding. The excised aorta from the mice analyzed in Figure 3 was opened longitudinally, followed by Oil Red O staining. **(A)** Representative pictures (upper panels) and percentage areas of intimal atherosclerotic plaque lesions of the aortic wall (bottom graph) are shown. Bar graphs represent means ± SD. The dots on bar graphs represent each individual data point [n=15: I^+L^- Eze (−) mice, n=8: I^+L^- Eze (+) mice, n=9: I^+L^+ Eze (−) mice, n=4: I^+L^+ Eze (+) mice, n=12: I^-L^- Eze (−) mice, n=4: I^-L^- Eze (+) mice, n=7: I^-L^+ Eze (−)

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mice, n=4: I^-L^+ Eze (+) mice]. $^{**}p<0.01$, significantly different by Dunnett's test comparing I^+L^+ Eze (−) mice, I^-L^- Eze (−) mice, and I^-L^+ Eze (−) mice with I^+L^- Eze (−) mice. $^{\dagger\dagger}p<0.01$, significantly different by Student's *t*-test comparing two groups [Eze (−) and Eze (+)] in each genotype. N.S., not significantly different between the groups. N.D., not detected. **(B and C)** Correlation of the percentage areas of intimal atherosclerotic plaque lesions with plasma concentrations of **(B)** cholesterol and **(C)** triglyceride. Correlation analysis was performed using Pearson's method. *R* value represents Pearson's correlation coefficient.

Figure 6. Schematic illustration of involvement of intestinal and hepatic NPC1L1 in the development/progression of dyslipidemia and atherosclerosis in LDLR mutant mice. Intestinal NPC1L1 is fundamentally involved in the development/progression of western diet-induced dyslipidemia and atherosclerosis via intestinal absorption of dietary lipids, including cholesterol. In addition to intestinal NPC1L1, hepatic NPC1L1 can exacerbate dyslipidemia and atherosclerosis, probably via the re-absorption of biliary lipids when intestinal NPC1L1 is functional.

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Figure 1

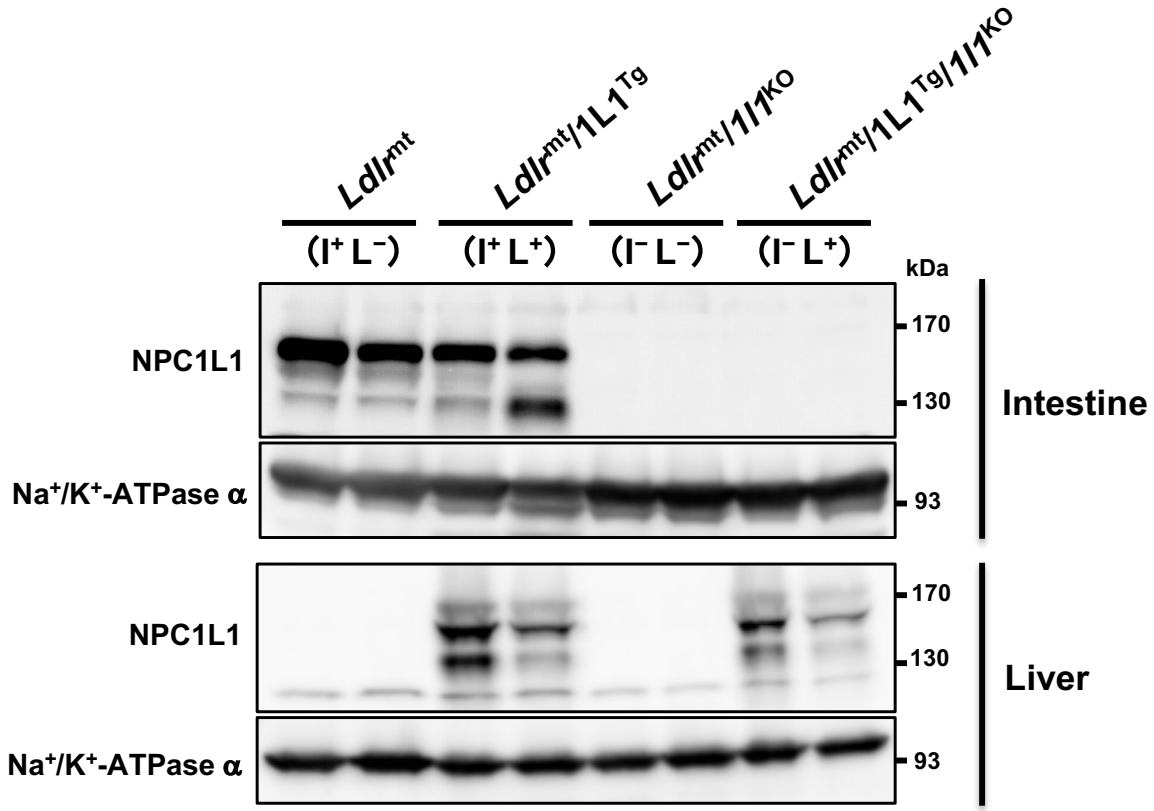

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Figure 2

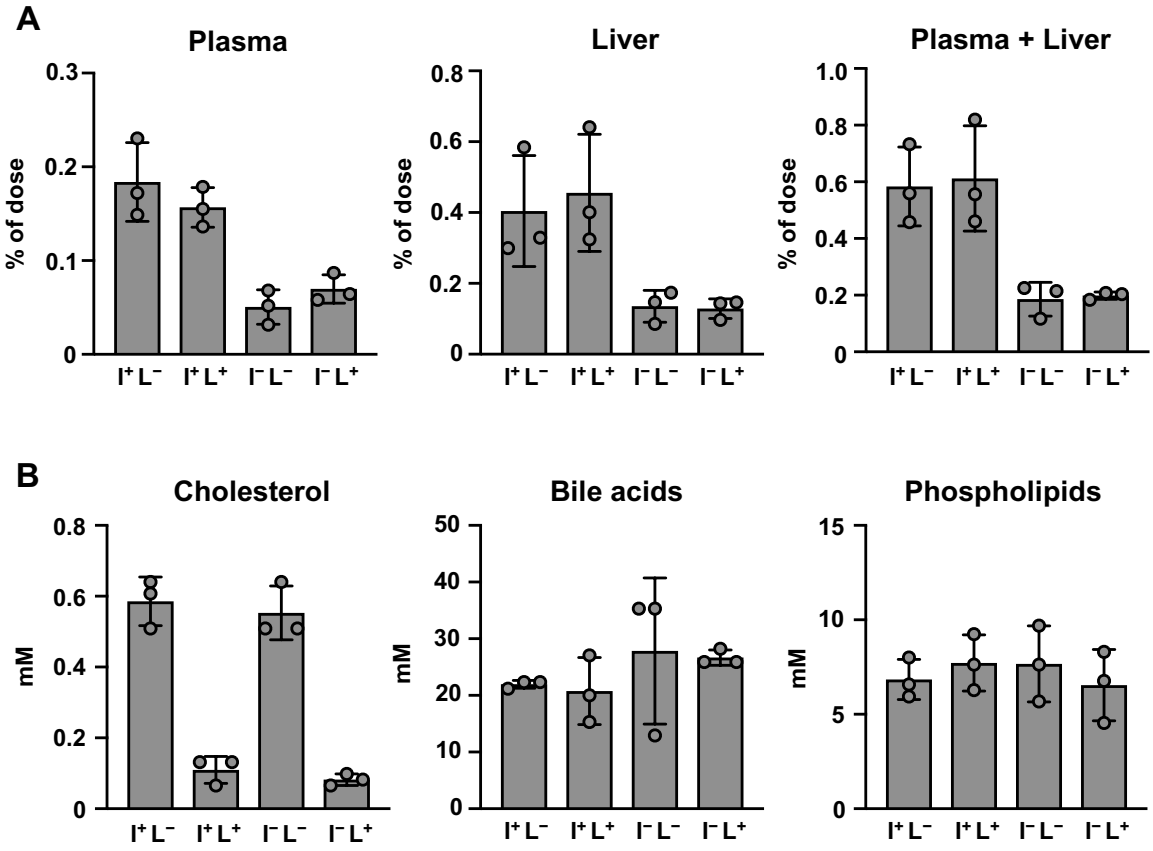

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Figure 3

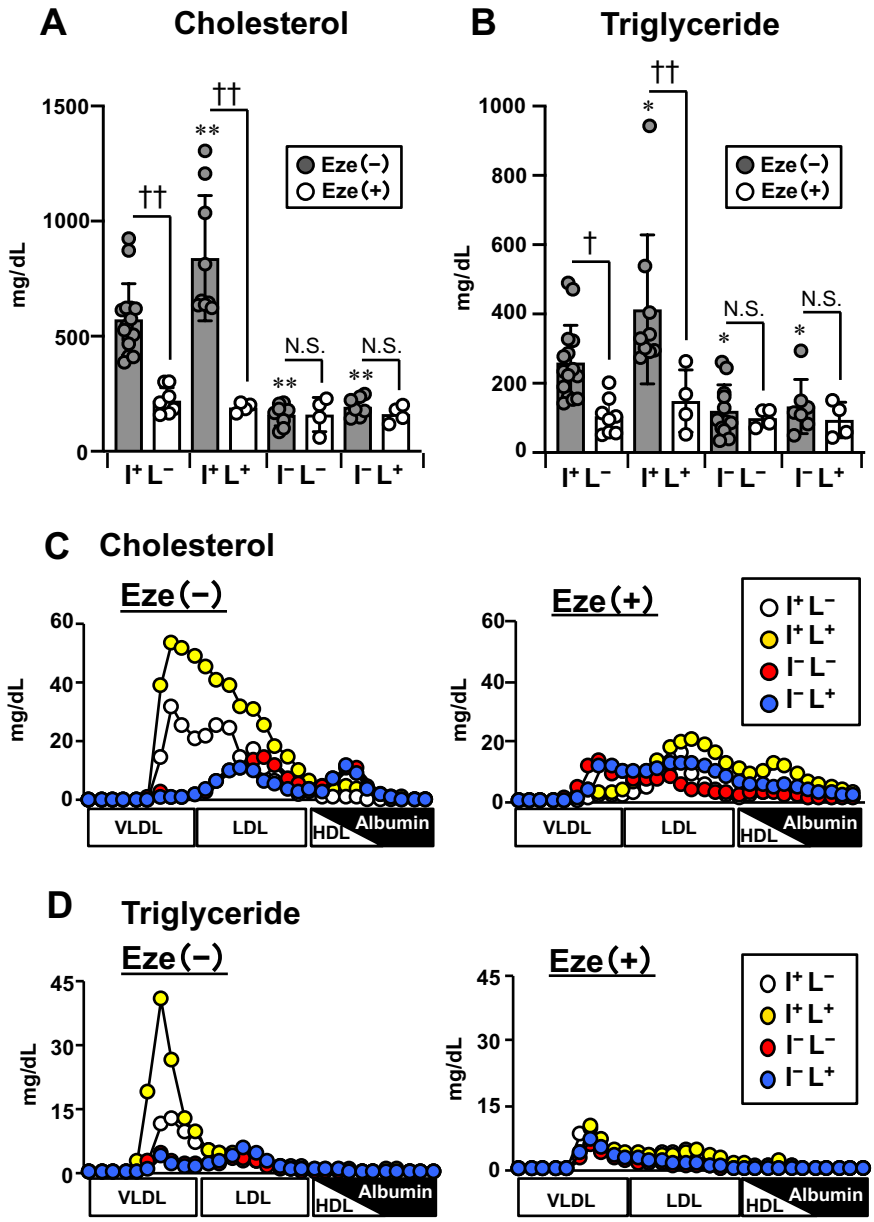

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Figure 4

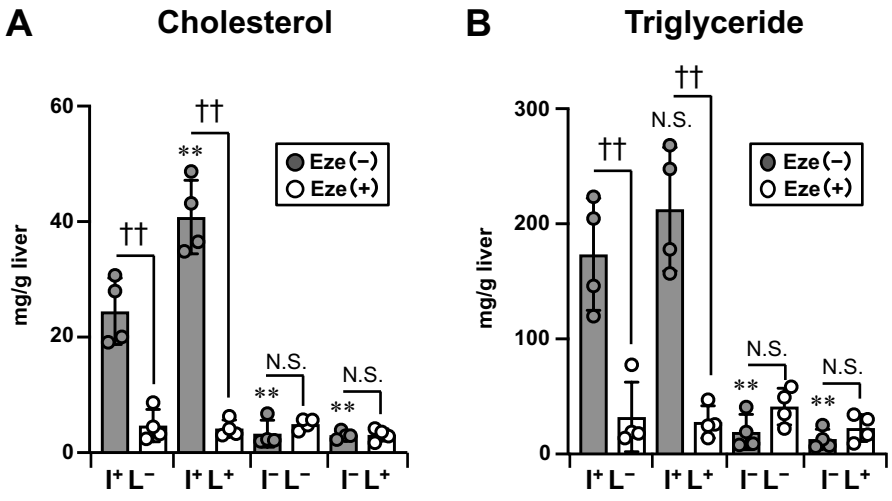

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Figure 5

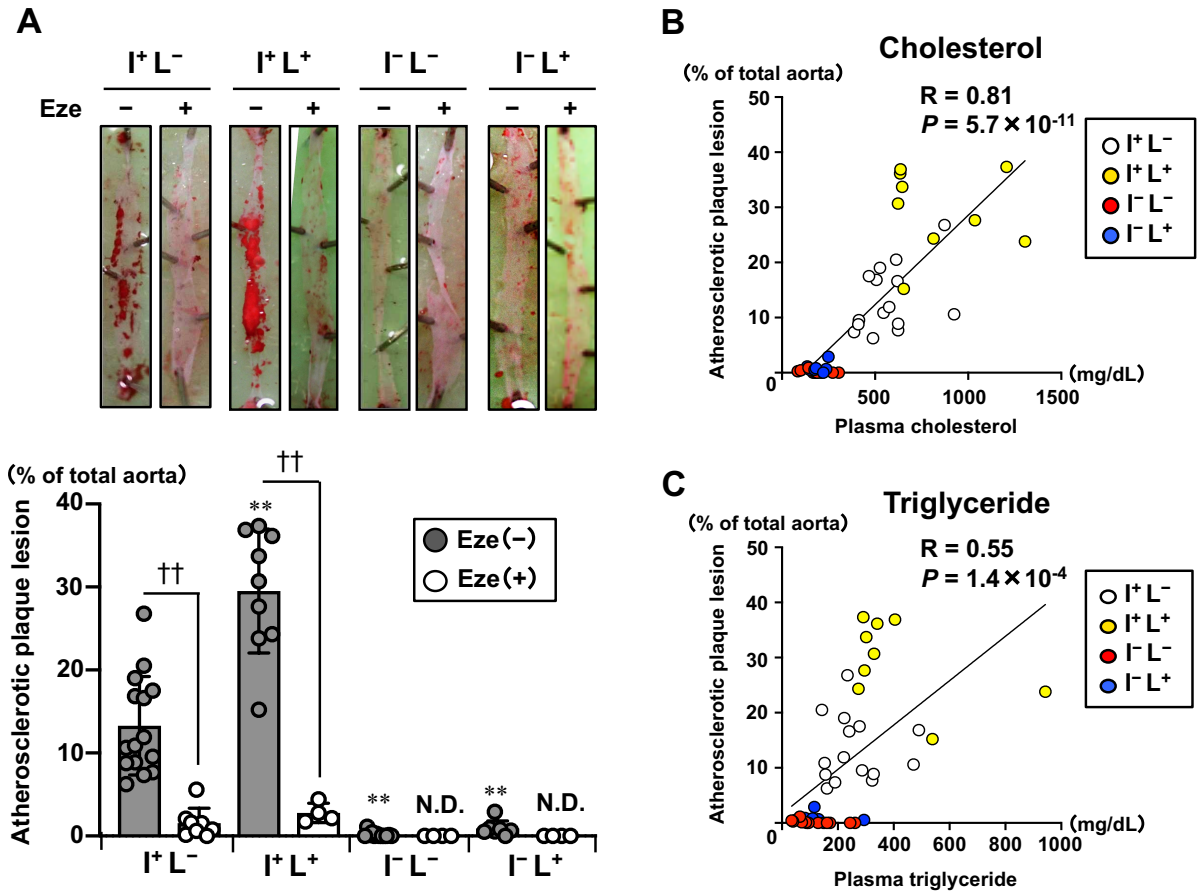
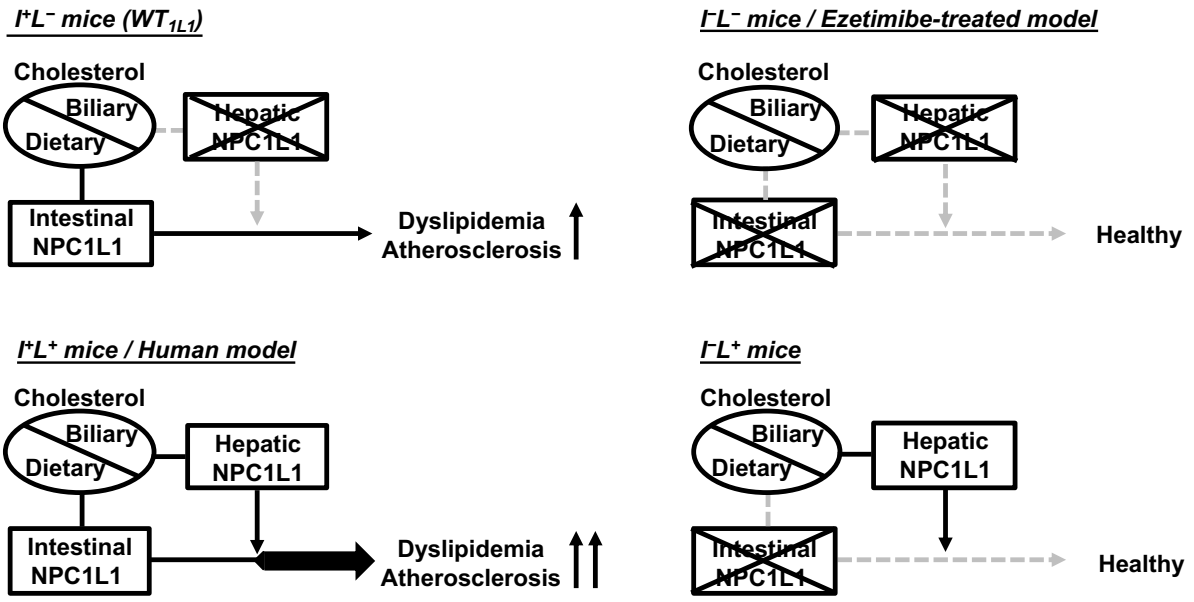


Figure 6


I+L− mice (WT1L1)
Cholesterol
Biliary
Dietary
Hepatic NPC1L1
Intestinal NPC1L1
Dyslipidemia
Atherosclerosis
I−L− mice / Ezetimibe-treated model
Cholesterol
Biliary
Dietary
Hepatic NPC1L1
Intestinal NPC1L1
Healthy
I+L+ mice / Human model
Cholesterol
Biliary
Dietary
Hepatic NPC1L1
Intestinal NPC1L1
Dyslipidemia
Atherosclerosis
I−L+ mice
Cholesterol
Biliary
Dietary
Hepatic NPC1L1
Intestinal NPC1L1
Healthy

Molecular Pharmacology

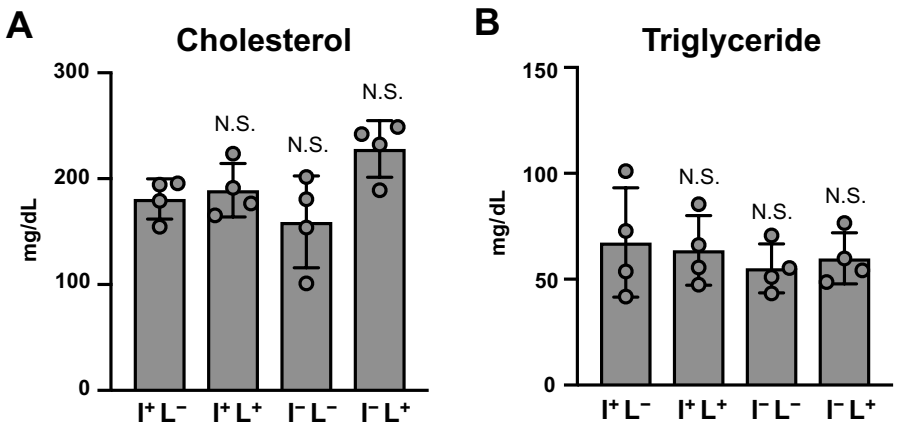
Hepatic expression of Niemann-Pick C1-Like 1, a cholesterol re-absorber from bile, exacerbates western diet-induced atherosclerosis in LDL receptor mutant mice.

Hideaki Yamamoto, Yoshihide Yamanashi, Tappei Takada, Shuang Mu, Yusuke Tanaka, Toko Komine, Hiroshi Suzuki.

Supplemental Table 1. Body weights of *NPC1L1* gene-modified LDLR mutant mice. Body weights of the generated 6-8-week-old male mice before and after 20 weeks of western diet (WD)-feeding in the absence or presence of ezetimibe (Eze) treatment. Data are expressed as mean ± SD.

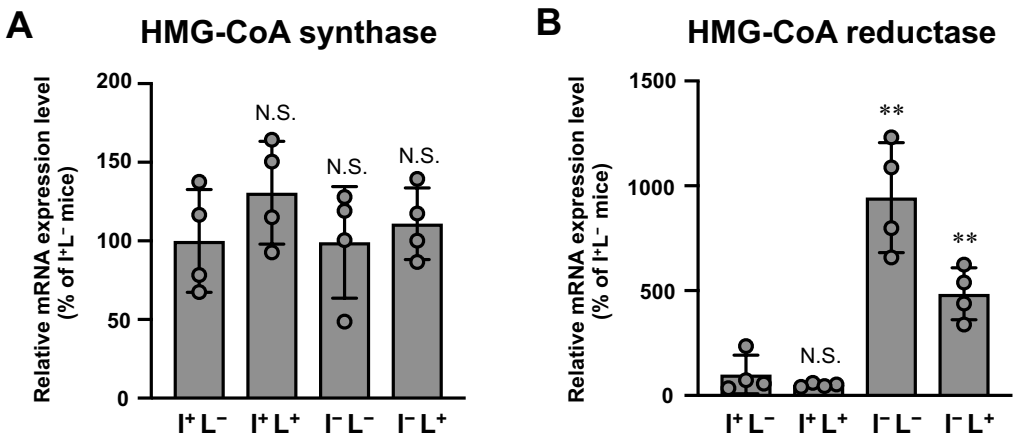
Mouse	Body weight (g)	
	WD feeding for 20 weeks	
	Before	After
I^+L^- Eze (−)	21.1 ± 1.0	45.7 ± 4.8
I^+L^- Eze (+)	21.6 ± 1.4	46.3 ± 2.0
I^+L^+ Eze (−)	20.7 ± 1.2	46.6 ± 1.2
I^+L^+ Eze (+)	21.5 ± 0.6	35.5 ± 4.0
I^-L^- Eze (−)	21.2 ± 1.0	35.1 ± 3.8
I^-L^- Eze (+)	21.0 ± 1.4	36.4 ± 2.4
I^-L^+ Eze (−)	21.0 ± 1.4	30.2 ± 1.6
I^-L^+ Eze (+)	20.0 ± 1.8	32.0 ± 6.0

Supplemental Figure 1



Supplemental Figure 1. Plasma lipid concentrations in *NPC1L1* gene-modified LDLR mutant mice before western diet feeding. (A) Cholesterol and **(B)** triglyceride concentrations in plasma from 6-8-week-old male mice before western diet feeding. Bar graphs represent means ± SD. The dots on bar graphs represent each individual data point (n=4). N.S., not significantly different from I^+L^- mice by Dunnett's test.

Supplemental Figure 2



Supplemental Figure 2. Hepatic mRNA expressions of genes involved in *de novo* cholesterol synthesis in *NPC1L1* gene-modified LDLR mutant mice after western diet feeding. Relative mRNA expressions of **(A)** 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, and **(B)** HMG-CoA reductase in the liver from male mice (initially 6-8-week-old) fed with a western diet for 20 weeks. Bar graphs represent means ± SD. The dots on bar graphs represent each individual data point (n=4). $^{**}p<0.01$, significantly different from I^+L^- mice by Dunnett's test. N.S., not significantly different.