Deficiency of Nardilysin in the Liver Reduces Serum Cholesterol Levels

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Nardilysin (NRDC) has been shown to be involved in post-translational histone modifications, in addition to enhancement in ectodomain shedding of membrane-anchored protein, which play significant roles in various pathophysiology, including glucose homeostasis, inflammatory diseases and cancer. The present study sought to determine roles of NRDC in the liver on lipid and lipoprotein metabolism. We established liver-specific NRDC deficient mice by use of NRDI floxed mice and albumin promoter-Cre recombinase (Cre) transgenic mice, and found that their serum low-density lipoprotein (LDL) cholesterol levels were significantly lower than those in control littermate mice. In the liver, LDL receptor (LDLR) mRNA expression was significantly upregulated, while inducible degrader of LDLR (IDOL) and microsomal triglyceride transfer protein (MTP) mRNA expression was significantly downregulated, in liver-specific NRDC deficient mice. Hepatic cell-surface LDLR expression levels were significantly elevated and serum pro-protein convertase subtilisin–kevin type 9 (PCSK9) levels were significantly reduced in mice with hepatic NRDC deficiency. In cultured hepatocytes, NRDC deficiency significantly reduced secreted PCSK9 and increased cell-surface LDLR expression. On the other hand, NRDC overexpression in cultured hepatocytes significantly increased secreted PCSK9 and lowered cell-surface LDLR expression. Thus, NRDC in murine hepatocytes appears to play key roles in cholesterol homeostasis, although the precise molecular mechanisms remain to be determined.

Key words nardilysin; cholesterol; low-density lipoprotein (LDL) receptor; pro-protein convertase subtilisin–kevin type 9 (PCSK9); inducible degrader of LDL receptor (IDOL)

INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) is a major cause of death in developed and developing countries worldwide. Dyslipidemia, especially elevated serum low-density lipoprotein (LDL) cholesterol (LDL-C) levels are one of the most potent risk factors for ASCVD. Reduction in serum cholesterol levels have been shown to be effective to prevent ASCVD[3]; however, serum LDL-C goal attainment was not necessarily desirable. Serum LDL-C levels are mostly regulated by endocytosis of LDL via hepatic LDL receptors (LDLR). Cell-surface expression of LDLR depends upon transcriptional activation by sterol responsible element binding protein 2 (SREBP2), as well as post-translational regulation (protein degradation) by pro-protein convertase subtilisin–kevin type 9 (PCSK9) and inducible degrader of LDL receptor (IDOL), an E3-ubiquitin ligase, which are activated by SREBP2 and liver X receptor (LXR), respectively. PCSK9 is a protein in circulating blood which binds to the LDLR on the plasma membrane surface, is taken up together with LDL particles, and then enhances the degradation by promoting the recruitment of the LDLR from endosomes to lysosomes. On the other hand, the IDOL promotes proteasome-dependent LDLR degradation. Among these, PCSK9 inhibitors, in addition to statins, have been established as strong tools to treat severe hypercholesterolemia. In addition to cell-surface expression of LDLR, serum LDL cholesterol levels appear to be modulated by very low density lipoprotein (VLDL) secretion from the liver via microsomal triglyceride transfer protein (MTP). In fact, a MTP inhibitor has been shown to be effective even in patients with the most severe types of hypercholesterolemia, such as homozygous familial hypercholesterolemia.10,11

Nardilysin (N-arginine dibasic convertase; NRDC), a metalloendopeptidase belonging to M16 family, has a molecular structure characterized by a highly acidic domain inside the enzymatic domain, which is widely expressed throughout the body, and is particularly strongly expressed in the liver, heart, testis, and lungs. NRDC was originally identified as a receptor for heparin-binding epidermal growth factor-like growth factor (HB-EGF). Subsequent studies have shown that NRDC plays diverse physiological roles depending on their subcellular localization. NRDC has been shown to promote ectodomain shedding of various cell-surface molecules, including HB-EGF, tumor necrosis factor alpha (TNF-α) and amyloid precursor protein (APP). Besides these extracellular functions, NRDC in the nucleus is involved in post-translational histone modifications, acting as a dimethyl-H3K4 (H3K4me2)-binding protein. NRDC interacts with NCoR/SMRT/HDAC3 corepressor on target genes. In brown adipose tissues, NRDC interactions with peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) alpha coactivator on Ucp1 enhancer repress transcription, and control thermogenesis. In pancreatic β-cell, NRDC interacts with islet-1 on Mafa enhancer activate transcription and regulates insulin secretion. In vivo, NRDC deficiency in mice

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showed growth retardation, hypomyelination, hypothermia and glucose intolerance.\(^{18,20-22}\) Furthermore, NRDC deficiency reduced hepatic fibrosis and inflammation in a dietary-induced non-alcoholic steatohepatitis,\(^{21}\) as well as gastric cancer progression, metaplasia and inflammation\(^{12,23}\) and autoimmune arthritis.\(^{20}\) in mice. NRDC also reduces acetylation of H3K9, increases H3K4me2, and controls cell cycle-related genes.\(^{27}\) In human colon cancer cells, NRDC associates with HDAC1, and NRDC overexpression and deficiency in mice suppresses and enhances p53 acetylation, and thereby promotes and reduces intestinal tumor growth, respectively.\(^{25}\)

Hepatocytes play central roles in lipid and lipoprotein metabolism, and appear to be involved in the development of metabolic diseases, including dyslipidemia. Previous studies using NRDC whole body deficient mice revealed that NRDC deficiency could suppress the progression of liver diseases in mice.\(^{21,26}\) In humans, high serum NRDC levels predict poor prognosis of hepatocellular carcinoma based upon viral hepatitis C,\(^{20}\) as well as intrahepatic cholangiocarcinoma.\(^{27}\) However, roles of hepatic NRDC in metabolic disorders have not yet been fully defined. The purpose of this study, therefore, was to investigate the roles of hepatic NRDC in metabolic diseases, focusing on cholesterol and lipoprotein metabolism.

MATERIALS AND METHODS

**Generation of Liver-Specific NRDC Deficient Mice**

To obtain liver-specific NRDC knockout (NRDC\(^{LKO}\)) mice, the Nrd1 floxed (NRDC\(^{f/f}\)) mice (Accession No. CDB1019K, http://www.clst.riken.jp/arg/mutant%20mice%20list.html), which contain a loxP site surrounding exon 1 of Nrd1 gene, were generated by gene targeting methods as previously described,\(^{19}\) and were crossed with albumin promoter-Cre recombinase (Cre) transgenic (Alb-Cre) mice in the C57BL/6J LKO mice, the obtain liver-specific NRDC knockout (NRDC\(^{f/f}\)) mice at 10–12 weeks of age by a two-step collagenase perfusion method as previously described,\(^{18}\) and were crossed with albumin promoter-Cre recombinase (Cre) transgenic (Alb-Cre) mice\(^{28}\) in the C57BL/6J strain. In genotyping PCR analysis, by use of mouse tail DNA, primers used for Cre and myogenin, as a control, were as follows: Cre sense, 5'-GAA CCTGAT GGA CATGTT CAGG-3', Cre antisense 5'-AGTGCC TCGAACGCTAGACCTGG-3', myogenin sense, 5'-TTACGG TCTCAGTGGACGC-3', myogenin antisense, 5'-TGGGCTGG TGTAGCCCTTA-3'.

**Animal Experiments**

NRDC\(^{LKO}\) and NRDC\(^{f/f}\) male mice were weaned at 4 weeks of age, and then were fed standard chow diet (MF; Oriental Yeast, Tokyo, Japan) and accessed water ad libitum. At 10–12 weeks of age, they were randomly divided into two groups per genotype and switched to high-fat and non-cholate (D12108C; Research Diets, U.S.A.) or cholesterol diet (HCD) containing 1.25% cholesterol, 40 kcal % fat and non-cholate (DI2108C; Research Diets, U.S.A.) or kept on the standard diet (SD). All the mice in this study were kept on the standard diet (SD). All the mice in this study were housed in a specific-pathogen free under controlled conditions of room temperature at 22°C and 12 h light–dark cycles. Their body weight and the amounts of food intake were measured weekly. After HCD or SD feeding for 20 weeks, their serum samples and liver tissue specimens were obtained. Tissue samples were quickly frozen by liquid nitrogen. Serum and tissue samples were kept frozen at −80°C until use. These study protocols were approved by the Ethics Committee for Animal Experiments of Kobe Gakuin University.

**Isolation and Cell Culture of Mouse Primary Hepatocytes**

Mouse primary hepatocytes were isolated from livers of NRDC\(^{LKO}\) and NRDC\(^{f/f}\) male mice at 10–12 weeks of age by a two-step collagenase perfusion method as previously described.\(^{29}\) In brief, after mice were anesthetized with isoflurane (Wako, Osaka, Japan), livers were perfused with prewarmed ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) buffer (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 5.6 mM glucose, 10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 0.5 mM EGTA, pH 7.5). Then, hepatocyte suspensions were obtained by perfusion of the liver with collagenase buffer containing 2 mg/mL collagenase type II (Worthington, PA, U.S.A.) in the EGTA buffer, and the undigested liver tissue was removed using 70 µm cell strainer (Falcon, Saitama, Japan). After isolation, hepatocytes were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (1.0 g/L glucose; Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO, U.S.A.), 100 U/mL penicillin, 100 µg/mL streptomycin and 0.3 mg/mL glutamine, and seeded on collagen type I-coated dishes (IWAKI, Shizuoka, Japan) at a density of 10⁴ cells/mL. A mouse hepatocyte cell line AML12 cells (CRL-2254) were purchased from American Type Culture Collection (Manassas, VA, U.S.A.) and cultured in DMEM and Ham’s F12 medium 1:1 medium (Nacalai Tesque) with 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium mixture (Roche, Basel, Switzerland), 40 ng/mL dexamethasone (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FBS at 37°C in 95% humidity and 5% CO₂. After incubation at 37°C for 24 h, the cells were washed with phosphate-buffered saline (PBS; Nacalai Tesque) twice. Then, the culture media were replaced with fresh culture media and the cell-conditioned media were collected after additional incubation for 24 h to measure PCSK9 secretion from cultured hepatocytes.

**Transient Transfections**

To generate V5-tagged NRDC expression vector, cDNA encoding full length of mouse NRDC was sub-cloned into pcDNA3.1/V5-histidine (His) vector,\(^{18}\) AML12 cells were seeded on 6-well plates and transfected with 1 µg of V5-tagged NRDC expression vector or empty vector using HilyMax transfection reagent (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. At 24 h after transfection, the culture media were replaced with fresh culture media, and the cell-conditioned media were collected after additional incubation for 24 h. To remove cells and precipitates, the cell-conditioned media were centrifuged and stored frozen at −110°C until the use for enzyme-linked immunosorbent assay (ELISA).

**RNA Extraction and Quantitative RT-PCR**

Total RNA was isolated and purified using RNAiso Plus Reagent (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions. Reverse transcription was carried out with 1 µg of total RNA using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) in accordance with the manufacturer’s instructions. Quantitative real-time PCR was performed by use of 7500 Fast Real-time PCR system (Applied Biosystems, Waltham, MA, U.S.A.) and specific genes were amplified by 40 cycles using THUNDERBIRD SYBR qPCR Mix (TOYOBO). Target gene expression was normalized for comparison by measuring the level of β-actin mRNA expression. All reactions were performed in triplicate. Specific primer pairs used for PCR were as follows: NRDC sense, 5'-ATGGATGGCCTTTCCCTTG-3'; NRDC antisense, 5'-CGCGAGGTTAGCTTTGC-3'.
Protein Extraction

Whole cell lysates were obtained from approximately 50 mg of the liver tissue and cultured hepatocytes by homogenation in radio immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) supplemented with complete mini protease inhibitor (Roche) followed by centrifugation at 14000 rpm for 10 min at 4 °C to remove debris. To obtain the plasma membrane fraction, approximately 50 mg of liver tissues and cultured hepatocytes were homogenized in buffer A (25 mM sucrose, 2 mM MgCl2, and 20 mM Tris–HCl, pH 7.6) supplemented with complete mini protease inhibitor, and were centrifuged at 2000×g for 10 min at 4 °C. The supernatants were ultracentrifuged at 120000×g for 1 h at 4 °C. The pellets were resuspended in buffer B (80 mM NaCl, 2 mM CaCl2, 50 mM Tris–HCl, pH 7.6) supplemented with complete mini protease inhibitor, and were centrifuged at 10000×g for 5 min at 4 °C. After centrifugation, the supernatants were used as the plasma membrane fraction. These protein samples were stored at −80 °C until the use for Western blot analysis. Protein concentrations of the samples were measured by bicinchoninic acid (BCA) protein assay kit (TaKaRa).

Western Blot Analysis

Individual or pooled plasma membrane samples (15–20 μg protein) from the liver, other organs or cultured hepatocytes were solubilized in lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher Scientific) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 8% polyacrylamide gel under denaturing and reducing conditions, then transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The electro-blotted membranes were blocked in phosphate-buffered saline-0.05% Tween 20 (PBS-T) containing 3% (w/v) non-fat dry milk for 1 h at room temperature and incubated with the primary antibody dissolved in PBS-T with 1% (w/v) non-fat dry milk for overnight at 4 °C. Primary antibodies used for immunoblotting were as follows: LDLR (0.14–0.35 μg/mL, ab-52818, Abcam), NRDC (1 : 2000) (#23), V5 (1.0 μg/mL, R960-25, Invitrogen) and β-actin (0.1–0.2 μg/mL, Wako).
After incubation with the primary antibodies, the membranes were washed three times with PBS-T, and then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (sc-2922, Santa Cruz) dissolved in PBS-T with 1% (w/v) non-fat dry milk for 1 h at room temperature. After washing three times with PBS-T, the immunoluminescence signals were visualized by Chemi-Lumi One (Nacalai Tesque) and detected by use of a VersaDoc 5000 MP (Bio-Rad). All specific band intensity was quantified by the Image J software.

Measurement of Serum Lipoprotein Cholesterol by HPLC and PCSK9 by Enzyme-Linked Immunosorbent Assay

Blood samples were collected from orbital veins of anesthetized mice after overnight fasting, and were centrifuged at 16,900 \( \times \) g for 5 min at 4 °C to obtain serum. Total and lipoprotein, including LDL sub-fraction, cholesterol levels in the sera were analyzed by HPLC (LipoSEARCH, Skylight Biotech, Akita, Japan). To determine PCSK9 levels in sera and cell-conditioned cultured media, specific ELISA for murine PCSK9 were performed by use of a mouse PCSK9 ELISA kit (DY3985; R&D systems). ELISA was carried out in triplicate.

Measurement of Cholesterol Levels in the Liver

To measure cholesterol levels in the liver, lipids were extracted by Folch’s methods as previously described.30 In brief, 50 mg of liver tissues were homogenized in chloroform/methanol (2:1 (v/v)) mixture using BioMasher (Nippi, Tokyo, Japan) and added distilled water. After centrifugation at 9000 \( \times \) g for 10 min at room temperature, the lower phase was collected and air-dried overnight. Concentrations of total cholesterol in the liver tissue were determined using LabAssay Cholesterol (Wako, Osaka, Japan) according to the manufacturer’s instructions. Liver cholesterol levels were normalized to the protein concentrations.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed by unpaired Student’s t-test. p Values less than 0.05 were considered as statistically significant.

RESULTS

Selective Deficiency in Hepatic NRDC Expression in Liver Specific NRDC Deleted (NRDC\(^{LKO}\)) Mice

Cre gene was selectively detectable in NRDC\(^{LKO}\) but not in the control NRDC\(^{EFT}\) mice, although the control myogenin gene was equally present, as confirmed by genomic PCR (Fig. 1A). qRT-PCR showed drastically reduced NRDC mRNA expression in livers of NRDC\(^{LKO}\) mice when compared to those in the control NRDC\(^{EFT}\) mice (1.1 ± 0.1 vs. 0.1 ± 0.01 arbitrary unit (a.u.), Fig. 1B). Western blot analyses showed remarkably reduced NRDC expression in livers of NRDC\(^{LKO}\) mice when compared to those in the control NRDC\(^{EFT}\) mice (10.1 ± 0.4 vs. 0.2 ± 0.01 a.u.). In other organs except for the liver, such as brain, kidneys, lungs and intestine, expression levels of NRDC were comparable (Fig. 1C). Primary cultured hepatocytes isolated from NRDC\(^{LKO}\) mice showed negligible NRDC mRNA (Fig. 1D) and protein (Fig. 1E) expression when compared to those isolated from control NRDC\(^{EFT}\) mice, as shown by qRT-PCR and Western blotting, respectively.

Comparison of Body Weight, Liver Weight and Food Intake between NRDC\(^{LKO}\) Mice and NRDC\(^{EFT}\) Mice

Weekly changes in the body weight are shown in NRDC\(^{LKO}\) and control NRDC\(^{EFT}\) mice fed SD or HCD (SD; NRDC\(^{EFT}\) n = 16, NRDC\(^{LKO}\) n = 12, HCD; NRDC\(^{EFT}\) n = 12, NRDC\(^{LKO}\) n = 11, *p < 0.05 (NRDC\(^{EFT}\) vs. NRDC\(^{LKO}\) mice fed HCD) (A). Amounts of food intake per body weight are shown in NRDC\(^{LKO}\) and control NRDC\(^{EFT}\) mice fed SD or HCD (SD; NRDC\(^{EFT}\) n = 16, NRDC\(^{LKO}\) n = 12, HCD; NRDC\(^{EFT}\) n = 12, NRDC\(^{LKO}\) n = 11) (B). Liver weight per body weight is shown in NRDC\(^{LKO}\) and control NRDC\(^{EFT}\) mice fed SD or HCD (SD; NRDC\(^{EFT}\) n = 16, NRDC\(^{LKO}\) n = 12, HCD; NRDC\(^{EFT}\) n = 12, NRDC\(^{LKO}\) n = 13) (C). Total cholesterol levels in the liver tissue are shown in NRDC\(^{LKO}\) and control NRDC\(^{EFT}\) mice fed SD or HCD (SD; NRDC\(^{EFT}\) n = 16, NRDC\(^{LKO}\) n = 12, HCD; NRDC\(^{EFT}\) n = 12, NRDC\(^{LKO}\) n = 13) **p < 0.01 (NRDC\(^{EFT}\) vs. NRDC\(^{LKO}\) mice fed HCD) (D). Values are indicated as mean ± S.E.M.
mice fed HCD showed significantly lower body weight than control NRDC^f/f mice fed HCD. The body weight was almost comparable between NRDC^LKO mice and control NRDC^f/f mice fed SD. Thus, hepatic NRDC deletion appeared to prevent body weight gain induced by HCD feeding. Food intake, however, was almost equal between NRDC^LKO mice and control NRDC^f/f mice (Fig. 2B), indicating that food preference or appetite changes was not the cause of the observed body weight differences. Similarly to the body weight, hepatic NRDC deletion significantly suppressed liver weight gain (Fig. 2C). Furthermore, in mice fed HCD, hepatic cholesterol content was not significantly different between HCD-fed NRDC^LKO and NRDC^f/f mice (p = 0.29, Fig. 2D).

**Effect of Hepatic NRDC Deficiency on Serum Total Cholesterol and LDL-C Levels**

Comparison of serum total cholesterol and LDL-C between NRDC^LKO mice and control NRDC^f/f mice is shown in Fig. 3. Serum total cholesterol (A), LDL-C (B), large LDL-C (C), medium LDL-C (D), small LDL-C (E) and very small LDL-C (F) levels in NRDC^LKO (n = 23) and control NRDC^f/f mice (n = 28) fed SD (n = 28) or HCD (n = 23) are shown (SD: NRDC^LKO: n = 12, NRDC^f/f: n = 16, HCD: NRDC^LKO: n = 11, NRDC^f/f: n = 12). Values are indicated as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 4. Relative Expression Levels of Genes Affecting Cholesterol Metabolism in NRDC^LKO Mice Fed SD or HCD**

Relative mRNA expression level of LDLR (A), IDOL (B) and MTP (C) in the liver tissue of NRDC^LKO compared to those in the control NRDC^f/f mice fed SD (NRDC^f/f n = 16, NRDC^LKO n = 12) or HCD (NRDC^f/f n = 7, NRDC^LKO n = 9) as determined by quantitative RT-PCR. Expression levels were normalized to β-actin expression levels. Values are indicated as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001.
tion, Fig. 3D), small LDL-C (SD: 1.3 ± 0.1 vs. 0.9 ± 0.1 mg/dL; 30.8% reduction, HCD: 3.1 ± 0.4 vs. 1.5 ± 0.1 mg/dL; 51.6% reduction, Fig. 3E) and very small LDL-C (1.0 ± 0.1 vs. 0.5 ± 0.1 mg/dL; 50.0% reduction, HCD: 2.6 ± 1.2 vs. 0.8 ± 0.2 mg/dL; 69.2% reduction, Fig. 3F) levels were significantly reduced in NRDC<sup>LKO</sup> mice than in control NRDC<sup>f/f</sup> mice fed SD or HCD. Thus, serum total cholesterol, LDL-C, and all of the LDL sub-fraction cholesterol levels were significantly lower in NRDC<sup>LKO</sup> mice than in control NRDC<sup>f/f</sup> mice. Among them, very small LDL-C, which appears to be the most atherogenic lipoprotein sub-fraction, showed the highest reduction levels by hepatic NRDC deficiency.

Relative Expression Levels of Genes Affecting Cholesterol Metabolism in NRDC<sup>LKO</sup> Mice Fed SD or HCD To explore molecular mechanisms involved in the reduced LDL-C levels in NRDC<sup>LKO</sup> mice, expression levels of genes affecting cholesterol levels were measured by qRT-PCR. Expression levels of LDLR mRNA (SD: 1.0 ± 0.1 vs. 1.2 ± 0.1 a.u., HCD: 1.0 ± 0.1 vs. 1.4 ± 0.1 a.u., <i>p</i> < 0.05, Fig. 4A) were significantly higher in NRDC<sup>LKO</sup> mice than those in control NRDC<sup>f/f</sup> mice fed SD or HCD. In contrast, IDOL (SD: 1.0 ± 0.1 vs. 0.8 ± 0.02 a.u., HCD: 1.0 ± 0.1 vs. 0.8 ± 0.1 a.u., <i>p</i> < 0.05, Fig. 4B) and MTP (SD: 1.0 ± 0.1 vs. 1.4 ± 0.1 a.u., HCD: 1.0 ± 0.1 vs. 0.7 ± 0.1 a.u., <i>p</i> < 0.05, Fig. 4C) mRNA levels were significantly lower in NRDC<sup>LKO</sup> mice than those in control NRDC<sup>f/f</sup> mice fed SD or HCD. Thus, these modulated gene expression patterns appeared to be responsible for reduced serum LDL-C levels.

Hepatic NRDC Regulates Hepatic LDL Receptor Expression To examine whether cell-surface LDLR protein expression is also induced in NRDC<sup>LKO</sup> mice, Western blotting of the plasma membrane fraction isolated from liver homogenates was carried out. As indicated in Fig. 5A, cell-surface LDLR protein levels were significantly elevated in NRDC<sup>LKO</sup> mice than those in control NRDC<sup>f/f</sup> mice fed SD (1.0 ± 0.2 vs. 0.3 ± 0.4 a.u., <i>p</i> < 0.05) or HCD (1.0 ± 0.3 vs. 1.4 ± 0.6 a.u., <i>p</i> < 0.05). We also have compared cell-surface LDLR protein expression levels in primary cultured hepatocytes between those isolated from NRDC<sup>LKO</sup> mice and control NRDC<sup>f/f</sup> mice. Representative images of the Western blotting are shown in Fig. 5B. Cell-surface LDLR protein expression was significantly increased in hepatocytes isolated from NRDC<sup>LKO</sup> mice when compared to those isolated from control NRDC<sup>f/f</sup> mice. To explore, furthermore, whether hepatic NRDC regulates cell-surface LDLR protein expression, cell-autonomously, without mediating other cell types, a murine hepatocyte cell line AML12 cells were transfected with V5-tagged NRDC expression vector, and cell-surface LDLR protein expression levels were measured. Overexpression of NRDC in AML12 cells resulted in much less cell-surface LDLR protein expres-

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![Graph A](image1.png)  
**Fig. 5. Hepatic NRDC Regulates Hepatic LDLR Expression**

LDLR protein expression levels were determined by immunoblot analysis in the plasma membrane fraction of the liver tissue of NRDC<sup>LKO</sup> and control NRDC<sup>f/f</sup> mice fed SD or HCD (SD: NRDC<sup>LKO</sup> 16, NRDC<sup>f/f</sup> 12, HCD: NRDC<sup>LKO</sup> 12, NRDC<sup>f/f</sup> 13). LDLR protein levels were normalized to β-actin protein levels. Relative values compared to those in the control NRDC<sup>f/f</sup> mice are shown. Values are indicated as mean ± S.E.M. *<i>p</i> < 0.05 (A). LDLR protein expression levels in the membrane fraction were determined by immunoblot analysis in primary cultured hepatocytes derived from NRDC<sup>LKO</sup> and control NRDC<sup>f/f</sup> mice at 8–10 weeks of age (B), as well as in AML12 cells transfected with V5-tagged NRDC expression vector (C). Representative images are shown (B, C).

![Graph B](image2.png)

![Graph C](image3.png)  
**Fig. 6. Hepatic NRDC Regulates PCSK9 Secretion**

Circulating serum PCSK9 levels are shown in NRDC<sup>LKO</sup> and control NRDC<sup>f/f</sup> mice fed SD or HCD (SD: NRDC<sup>LKO</sup> 16, NRDC<sup>f/f</sup> 12, HCD: NRDC<sup>LKO</sup> 12, NRDC<sup>f/f</sup> 13) (A). Secreted PCSK9 levels in cell-conditioned media of primary cultured hepatocytes derived from NRDC<sup>LKO</sup> and control NRDC<sup>f/f</sup> mice at 8–10 weeks of age (a = 4 per genotype) are shown (B). Secreted PCSK9 levels in cell-conditioned media of AML12 cells transfected with V5-tagged NRDC expression vector or mock-transfected are shown (a = 4 each) (C). PCSK9 concentrations were determined by ELISA. Values are indicated as mean ± S.E.M. *<i>p</i> < 0.05, ***<i>p</i> < 0.001.
sion (Fig. 5C), thus indicating the cell-autonomous effects of NRDC on cell-surface LDLR protein expression in hepatocytes without mediating other cell types.

**Hepatic NRDC Regulates PCSK9 Secretion** Since cell-surface expression of LDLR is also regulated by PCSK9, in addition to LDLR gene expression, we have explored whether NRDC regulates PCSK9 levels. In mice, serum PCSK9 levels were significantly ($p < 0.001$) less in NRDC$^{LKO}$ mice than those in control NRDC$^{f/f}$ mice (SD; 214.5 ± 8.1 vs. 165.4 ± 9.1 ng/mL, HCD; 298.1 ± 37.0 vs. 157.9 ± 20.4 ng/mL, Fig. 6A). In primary cultured hepatocytes, as shown in Fig. 6B, concentrations of PCSK9 were significantly ($p < 0.001$) lower in cell-conditioned media isolated from NRDC$^{LKO}$ mice (84.7 ± 7.1 ng/mL) than in those isolated from control NRDC$^{f/f}$ mice (119.6 ± 7.3 ng/mL). Furthermore, PCSK9 concentrations in cell-conditioned media isolated from AML12 cells were significantly increased by overexpression of NRDC (17.4 ± 0.9 vs. 19.7 ± 0.7 ng/mL, $p < 0.05$, Fig. 6C).

**DISCUSSION**

In this study, we investigated the roles of hepatic NRDC in the regulation of lipid metabolism, especially lipoprotein cholesterol metabolism. We found that serum total cholesterol and LDL-C levels were significantly reduced by hepatic NRDC deficiency. These reductions in serum cholesterol levels appeared to be due to increased uptake of circulating LDL into the liver by enhanced cell-surface LDLR expression resulting from both induced expression of LDLR and increased LDLR recycling to the cell-surface caused by reduced PCSK9 secretion in hepatocytes. LDLR gene transcription depends upon SREBP2 activation; therefore, post-translational histone modifications by NRDC may somehow activate SREBP2 and suppress secretion of PCSK9, although the precise molecular mechanisms remain to be clarified. In fact, PCSK9 inhibitors, such as evolocumab and alirocumab, in addition to statins, have been shown to have potent LDL-C-lowering effects, in clinical practice, in patients with extremely high serum LDL-C levels. 8,32)

In patients with LDLR-negative homozygous familial hypercholesterolemia, who are resistant to treatment with statins or PCSK9 inhibitors, lomitapide, an MTP inhibitor, has been shown to be effective to lower serum LDL-C levels. 10,11) Furthermore, it appears to be expected that reduced expression of IDOL may also synergistically contribute to the increased cell-surface expression of LDLR and may thereby lower serum LDL-C levels. 8,33) Interestingly, transcription of both IDOL 8) and MTP 34) gene transcription appears to depend upon activation LXR. Therefore, epigenetic effects of NRDC in the nucleus, as shown in previous studies, 16,17) might suppress LXR activation. These points should further be elucidated in future studies. Transcription of PCSK9, as well as LDLR, gene depends upon activation of SREBP2. Hepatic NRDC deficiency, in this study, induced LDLR mRNA expression; however, hepatic PCSK9 secretion was suppressed by hepatic NRDC deletion both in mice in vivo and in cultured cells. Thus, NRDC may have more positive impacts on secretion than production of PCSK9. Previous studies have shown that PCSK9 secretion appeared to depend upon sortilin 1 (SORT1) 35) and an endoplasmic reticulum (ER) cargo receptor SURF4 36); therefore, NRDC may affect expression or functions of SORT1 and/or SURF4. These points should be clarified in future studies. Furthermore, in mice with impaired LXR alpha phosphorylation, similarly to hepatic NRDC deficiency, decreased serum cholesterol levels and increased LDLR mRNA expression were observed under the condition of high cholesterol feeding. 37) Thus, hepatic NRDC deficiency may impair LXR activation and/or its downstream signals, including H3K27Ac, and thereby suppress expression of LXR-dependent genes, 37) although additional studies are necessary. In addition, it is known that LXR also plays important roles in fatty acid metabolism. 36) Suppression of the body weight gain in liver-specific NRDC-deficient mice fed HCD, as observed in this study, might be due to decreases in the amounts of body fat resulting from reduced hepatic lipogenesis caused by inhibition of LXR-dependent SREBP1c activation. Furthermore, among LDL sub-fractions, very small LDL-C showed the highest reduction levels by hepatic NRDC deficiency in mice. This may due to reduced lipogenesis and reduced TG levels, as shown in humans. Therefore, hepatic NRDC may also be involved in triglyceride metabolism in addition to cholesterol. These points also should be clarified in the future. However, it is well-known that molecular mechanisms involved in lipoprotein and cholesterol metabolism are not totally identical between humans and mice. For instance, cholesteryl ester transfer protein (CETP) is present in humans and but not in mice. Therefore, high density lipoprotein cholesterol (HDLC) is more abundant in mice than in humans, and LDL-C levels are higher in humans than in mice. Thus, the observed results in mice, in this study, may not readily be translated into humans, and thus require further studies in other species.

In summary, the present study indicated that hepatic NRDC appears to play important roles in cholesterol homeostasis and lipoprotein metabolism by modulating multiple molecular mechanisms. Therefore, hepatic NRDC may be one of integral regulator of cholesterol metabolism, and thus may provide novel therapeutic targets in treatment of dyslipidemia.

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**Conflict of Interest** The authors declare no conflict of interest.

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