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A new system to evaluate characteristics of Niemann-Pick C1 Like 1-mediated cholesterol transport using *Xenopus laevis* oocytes

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Abbreviations: ANOVA: Analysis of variance, BLT-1: Block lipid transport-1, CD36: Cluster determinant 36, cRNA: complementary RNA, DEPC: Diethyl pyrocarbonate, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, IgG: Immunoglobulin G, MDCK: Madin-Darby canine kidney, NPC1L1: Niemann-Pick C1 Like 1, PCR: Polymerase chain reaction, PdI: Poly dispersity index, RT: Reverse transcription, SR-BI: Scavenger receptor class B type I, TBS-T: Tris buffered saline with Tween 20

Abstract

Niemann-Pick C1 Like 1 (NPC1L1) is known to be involved in the intestinal absorption of cholesterol, but the detailed molecular mechanism of transport has not been elucidated. In this study, the characteristics of cholesterol transport via NPC1L1 were investigated using a Xenopus laevis oocyte expression system in addition to a conventional cell line with stable expression. The transport activity of cholesterol uptake was increased in NPC1L1-overexpressed Madin-Darby canine kidney (MDCK) cells compared with that in mock cells, but MDCK cells expressed endogenous NPC1L1 and had high cholesterol transport activity. It is therefore considered difficult to evaluate the characteristics of NPC1L1-mediated cholesterol uptake using MDCK cells. On the other hand, cRNA-injected oocytes expressed NPC1L1 on the cell membrane after culturing for 5-6 days. The transport activity of cholesterol uptake was increased in NPC1L1 cRNA-injected oocytes compared with that in water-injected oocytes. In addition, the uptake of cholesterol was decreased in the presence of ezetimibe, an NPC1L1 inhibitor, in cRNA-injected oocytes but not in control oocytes, indicating that endogenous NPC1L1 does not function in oocytes. Furthermore, cholesterol uptake was substantially decreased in NPC1L1 L216A cRNA-injected oocytes compared with that in NPC1L1 cRNA-injected oocytes, indicating that leucine at position 216 of NPC1L1 is important for cholesterol transport and that an oocyte expression system is useful for mutant analysis. The results indicate that the oocyte expression system is useful for

evaluating the characterisctics of NPC1L1-mediated cholesterol transport and may contribute to the elucidation of the detailed molecular mechanism of cholesterol transport via NPC1L1.

1. Introduction

Dietary cholesterol has low solubility, and the process of its absorption from the gastrointestinal tract involves emulsification of food by bile, digestion by lipase and solubilization in mixed micelles composed of bile salts, phospholipids and monoacylglycerol [1]. Niemann-Pick C1 Like 1 (NPC1L1), which is expressed in small intestinal epithelial cells, has been identified as a transport carrier involved in this absorption process [2]. NPC1L1, which has 13 transmembrane domains, is highly expressed in the small intestine and liver, and it was discovered as a homologue of Niemann-Pick C1 and found to be involved in cholesterol transport in intracellular vesicles [3,4]. NPC1L1 is involved in cholesterol absorption in the small intestine and cholesterol reabsorption from bile duct in the liver, and it contributes to the maintenance of cholesterol homeostasis [5,6]. In addition, it has been shown that a deficiency of NPC1L1 prevents atherosclerosis in apolipoprotein E-null mice and that hepatic expression of NPC1L1 exacerbates western diet-induced atherosclerosis in low-density lipoprotein receptor mutant mice [7,8]. NPC1L1 is also known as a target molecule for the cholesterol absorption inhibitor ezetimibe [9-11]. Ezetimibe is a drug that binds to NPC1L1 and inhibits its function, and it has been reported to reduce cholesterol absorption by 54% in patients with hypercholesterolemia compared with the level of absorption in a placebo group [12]. However, despite the clinical importance, the detailed

molecular mechanism of cholesterol transport via NPC1L1 has not been elucidated.

Caco-2 and Madin-Darby canine kidney (MDCK) II cells overexpressing NPC1L1 have been used for evaluating the function of NPC1L1 [13,14]. Caco-2 cells and MDCK II cells form brush borders and tight junctions similar to those formed by small intestinal epithelial cells in monolayer culture and are widely used as model cells for intestinal absorption. However, although cultured cells are easy to handle, it may be difficult to evaluate the characteristics of NPC1L1-mediated cholesterol transport because of the expression of endogenous NPC1L1. McA-RH7777 cells have also been used to evaluate the function of NPC1L1 [15]. However, the transport of plant sterols, a physiological substrate of NPC1L1, is not observed in this system.

Therefore, in this study, in addition to a conventional cell line with stable expression of NPC1L1, we developed a new system for evaluation of NPC1L1-mediated cholesterol uptake using *Xenopus laevis* oocytes. *Xenopus laevis* oocytes have been widely used as an expression system in transporter research because the expression of only a single transporter on the cell membrane can be induced by directly injecting cRNA into the cells. In addition, since this expression system has low endogenous transport activity, it may be applicable to mutant analysis. It has been reported that cholesterol bound to the N-terminal domain of NPC1L1 and that cholesterol binding ability was markedly reduced in mutants, especially in mutants in which leucine at position 216 of NPC1L1 was substituted with alanine [16]. Hence,

in this study, we also investigated the effect of L216A mutation on cholesterol uptake using the *Xenopus laevis* oocyte expression system.

2. **Materials and Methods**

2.1. Chemicals and Reagents

[1α, 2α, -3H (N)] Cholesterol was purchased from PerkinElmer, Inc. (Boston, MA). Cholesterol and sodium taurocholate were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). L-α-Phosphatidylcholine was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Isopropyl myristate was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Ezetimibe ((4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-4S-(4-hydroxyphenyl)-2-azetidinone) was purchased from LKT Laboratories, Inc. (St. Paul, MN). BLT-1, known as an SR-BI inhibitor, was purchased from Sigma-Aldrich Co., LLC (St. Louis, MA). All other reagents were of the highest grade available and were used without further purification.

2.2. Cell culture

MDCK cells were purchased from American Type Culture Collection (Manassas, VA). MDCK cells (passage number: 96-106) were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., LLC, St. Louis, MA) with 10% (v/v) fetal bovine serum (Biosera, Inc., Nuaillé, France), 100 IU/mL penicillin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 100 µg/mL streptomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and were grown in an atmosphere of 5% (v/v) CO_2 at 37˚C.

2.3. Animals

Xenopus laevis frogs were purchased from Hokudo (Sapporo, Hokkaido, Japan). The Frogs were kept in a water tank at 18˚C before the experiments. Studies using *Xenopus laevis* frogs have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Permission for this study was obtained from the Committee on Animal Experimentation, Hokkaido University.

2.4. Preparation of an emulsion and a mixed micelle

The emulsion was prepared as described previously [17]. The final composition of the emulsion was 2 µM cholesterol, 0.5 mM sodium taurocholate and 1% (v/v) isopropyl

myristate with or without 50 μM ezetimibe.

A mixed micelle was prepared according to the method described previously with some modifications [13,18]. In brief, cholesterol and [^{3}H]-cholesterol dissolved in ethanol, L-α-phosphatidylcholine dissolved in methanol, and sodium taurocholate dissolved in 95% (v/v) ethanol were mixed with or without ezetimibe or BLT-1, and the solvent was evaporated under N_2 gas. The dried residue was dispersed in transport buffer (25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, adjusted to pH 7.5 with Tris) or ND96 buffer (5 mM HEPES, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$, adjusted to pH 7.4 with Tris) and sonicated in a bath sonicator (SND, Nagano, Japan) for 30 min. The micellar solution was stirred at 37˚C for 1 h and filtrated through 0.22 μm polytetrafluoroethylene membranes (Recenttec K.K., Tokyo, Japan) before the experiment.

The average particle diameters of emulsions and mixed micelles were measured using a quasielastic light scattering method (Zetasizer Nano ZS; Malvern Panalytical Ltd., Malvern, England).

2.5. Preparation of NPC1L1-overexpressed MDCK cells

A human NPC1L1 clone was purchased from BioCat GmbH (Heidelberg, Germany). The coding sequence of NPC1L1 cDNA (Genbank accession number: AY437865) was

inserted into the pMAM2-BSD vector (Funakoshi Co., Ltd., Tokyo, Japan) and transfected into MDCK cells with lipofectamine reagent (Thermo Fisher Scientific Inc., Waltham, MA). Then NPC1L1-overexpressed MDCK cells were cloned after selection by culturing for 3 days in the presence of 9 μg/mL blasticidin S (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The L216A mutant of NPC1L1 was generated by site-directed mutagenesis and transfected into MDCK cells.

2.6. Preparation of NPC1L1-overexpressed oocytes

NPC1L1-overexpressed oocytes were prepared as described previously with minor modification [19]. Briefly, human NPC1L1 complementary RNA was synthesized from a linearized plasmid using a mMESSAGE mMACHINE kit (Ambion, Austin, TX). For optimizing the yield of long transcripts, 1 μL of GTP solution was added according to the manufacturer's protocol. Mature oocytes from *Xenopus laevis* were isolated by treatment with 1 mg/mL collagenase (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The oocytes were manually defolliculated and maintained at 16˚C in Barth's buffer (88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 0.05 mg/mL gentamicin and 10 mM HEPES, adjusted to pH 7.4 with Tris). On the following day, the oocytes were microinjected with either 30 nL DEPC-treated water containing cRNA or 30

nL DEPC-treated water alone. The injected oocytes were incubated in Barth's buffer for 3-6 days at 16˚C.

2.7. RT-PCR analysis

Total RNA was extracted from MDCK cells using RNA iso Plus (Takara Bio Inc., Shiga, Japan). cDNA was synthesized from total RNA using a ReverTra Ace qPCR RT Kit (TOYOBO Co., Ltd., Osaka, Japan). PCR was performed using SapphireAmp Fast PCR Master Mix (Takara Bio Inc., Shiga, Japan). Each experiment was conducted according to the manufacturer's protocol. The sequences of the specific primers used in PCR are listed in Table 1. The sequences of human NPC1L1 primers were the same as those described previously [17]. The PCR products were subjected to electrophoresis on a 2% (v/v) agarose gel and then stained by ethidium bromide.

2.8. Western blot analysis

For preparation of a total cell lysate of MDCK cells, the cell pellet was resuspended in lysis buffer (1.06 mM KH_2PO_4, 154 mM NaCl, 2.97 mM Na_2HPO_4, 1% (v/v) Triton X-100 and 0.1% (w/v) SDS) containing protease inhibitor cocktail (F. Hoffmann-La Roche Ltd.,

Basel, Switzerland). The suspension was incubated for 5 min on ice and was sonicated in a bath sonicator for 15 min at 4˚C. Then the suspension was centrifuged at 21,500×g for 15 min at 4˚C. The supernatant was used for the experiment as a total cell lysate.

For preparation of a crude membrane fraction of *Xenopus laevis* oocytes, 20 oocytes in homogenization buffer (0.55 M sucrose, 3.45 mM NaH_2PO_4 and 14.72 mM Na_2HPO_4) containing protease inhibitor cocktail were homogenized with an ultrasonic homogenizer. Then the suspension was centrifuged at 3,000×g for 15 min at 4˚C. After removal of lipids on the water surface, the supernatant was transferred to an ultracentrifuge tube. The supernatant in the ultracentrifuge tube was centrifuged at 48,000×g for 1 h at 4˚C. After removal of the supernatant, the pellet was resuspended in homogenization buffer with a vortex mixer. The suspension was used for the experiment as a crude membrane fraction.

The samples were diluted 2-fold with loading buffer (0.125 M Tris, 4% (w/v) SDS, 10% (w/v) sucrose, 0.01% (w/v) bromophenol blue and 10% (w/v) 2-mercaptoethanol), and 7.5 μg of protein was separated on an 8.4% (w/v) SDS-polyacrylamide gel with a 4.8% (w/v) stacking gel. Proteins were transferred electrophoretically to polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Buckinghamshire, England) at 90 mA for 120 min. The membranes were blocked for 1 h with TBS-T (20 mM Tris, 500 mM NaCl and 0.1% (v/v) Tween 20, adjusted to pH 7.5 with HCl) containing 5% (w/v) skim milk (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After washing with TBS-T, the membranes were

incubated overnight at 4˚C with primary antibodies: mouse anti-NPC1L1 monoclonal antibody (lot number: J2416, Santa Cruz Biotechnology, Inc., Dallas, TX) diluted 1:1000 and rabbit anti-NPC1L1 polyclonal antibody (lot number: A89884, Sigma-Aldrich Co., LLC, St. Louis, MA) diluted 1:1500. After washing with TBS-T, the membranes were incubated for 1 h at room temperature with secondary antibodies: horseradish peroxidase-conjugated goat anti-mouse IgG (SeraCare Life Sciences, Inc., Milford, MA) diluted 1:10000 and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX) diluted at 1:10000. After washing the membranes with TBS-T, chemiluminescence was observed by exposure to X-ray film in a darkroom according to the protocol instructions of Immunostar Zeta (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.9. Immunohistochemistry

Immunohistochemical analysis was performed according to the method described previously with some modifications [20]. Oocytes were fixed in 10% (v/v) formalin neutral buffer solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The fixed oocytes were embedded in paraffin and probed with rabbit anti-NPC1L1 antibody diluted 1:50 followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Inc., West Grove, PA) diluted 1:200. The samples were then

visualized by using a confocal laser scanning microscope (FV10i, Olympus Corporation, Tokyo, Japan).

2.10. [^{3}H]-cholesterol uptake assay

For uptake study in MDCK cells, cells were seeded at a density of 5.0×10^4 cells/well on a 24-well plastic plate (Corning Incorporated, Corning, NY) and cultured for 4 days. Before the experiment, cells were incubated in a medium containing 4 μg/mL dexamethasone (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 48 h. After removal of the medium, the cells were preincubated with transport buffer for 1 h at 37˚C. After removal of the transport buffer, the cells were incubated with 20 nM of emulsified or micellar [^{3}H]-cholesterol, 0 or 50 μM ezetimibe and 0 or 20 nM BLT-1 for the indicated time at 37˚C. At the end of the incubation period, the cells were washed three times with ice-cold transport buffer containing 1 mM sodium taurocholate. Then the cells were solubilized in 1% (w/v) SDS in 0.2 M NaOH. The radioactivity in the cell lysate was measured by a liquid scintillation counter (LSC-5100, Hitachi Aloka Medial, Ltd., Tokyo, Japan) to determine the uptake of cholesterol. For normalization, the protein concentration was determined by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA).

For uptake study in oocytes, 5-7 oocytes were preincubated with ND96 buffer for 30

min at 25˚C. After removal of the ND96 buffer, the cells were incubated with 20 nM of micellar [^{3}H]-cholesterol and 0 or 25 μM ezetimibe for the indicated time at 25˚C. At the end of the incubation period, the cells were washed three times ice-cold ND96 buffer. At this point, injured or dying oocytes were excluded from the analysis. Then the cells were solubilized in 4% (w/v) SDS in 0.2 N NaOH. The radioactivity in the cell lysate was measured by a liquid scintillation counter to determine the uptake of cholesterol.

2.11. Statistical analysis

Mean values in two groups were compared by the unpaired two-tailed t-test. Mean values in more than two groups were compared by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test. In two-way factorial ANOVA analysis, if the interaction between two factors was statistically significant, multiple comparisons of all groups were performed. Otherwise, multiple comparisons between levels of factors with statistically significant differences were performed. Variability around the mean in each group was described as S.D. All statistical analyses were performed using ORIGIN 2019 (OriginLab Corporation, Northampton, MA). Statistical significance was defined as $p<0.05$.

3. Results

3.1. Properties of emulsions and mixed micelles

Cholesterol that reaches the gastrointestinal tract is dispersed as an emulsion by bile and then digested by lipase and absorbed in the form of mixed micelles [1]. The average particle size and PdI value of the emulsion prepared in this study were 206.7 ± 2.82 nm and 0.18 ± 0.022, respectively (Table 2). The average particle size of the mixed micelles prepared in this study was smaller than that of the emulsions, while the PdI value of the mixed micelles was larger than that of the emulsions, indicating that they were polydispersed. The type of buffer in which the mixed micelles were dispersed did not affect the particle size or PdI value. In addition, there was no change in the average particle size or PdI value in the presence or absence of ezetimibe and BLT-1 (data not shown).

3.2. Evaluation of the characteristics of NPC1L1-mediated cholesterol transport using MDCK cells

Human NPC1L1 cDNA was transfected into MDCK cells, and the expression of human NPC1L1 in isolated clones was confirmed by RT-PCR and Western blot analysis (Fig. 1A, B). As shown in Fig. 1B, a thin band was also observed in the mock lane.

To analyze the function of NPC1L1, a [^{3}H]-cholesterol uptake assay was performed using NPC1L1-overexpressed MDCK cells (NPC1L1 cells). There was no difference in emulsified cholesterol uptake between pMAM2-BSD vector-transfected MDCK cells (mock cells) and NPC1L1 cells, and no inhibition by ezetimibe was observed (Fig. 1C). On the other hand, the transport activity of micellar cholesterol was increased in NPC1L1 cells compared with that in mock cells (Fig. 1D). In addition, the uptake of cholesterol was decreased in the presence of ezetimibe in NPC1L1 cells. In mock cells, the uptake of cholesterol was also decreased in the presence of ezetimibe. Next, the time course of cholesterol uptake was observed. The uptake of cholesterol was increased in a time-dependent manner in both mock and NPC1L1 cells (Fig. 1E).

To obtain a better understanding of the characteristics of NPC1L1-mediated cholesterol transport, NPC1L1 L216A-overexpressed MDCK cells (NPC1L1 L216A cells) were prepared, and the effect of L216A mutation on cholesterol uptake was investigated. The expression of NPC1L1 L216A was confirmed by Western blot analysis (Fig. 1F). A clone that showed the same level of expression as that of the wild type was used for the uptake assay. The uptake of cholesterol in NPC1L1 L216A cells was intermediate between that in mock cells and that in NPC1L1 cells (Fig. 1G).

3.3. Contribution of transport pathways other than NPC1L1 to uptake of cholesterol into

MDCK cells

SR-BI and CD36 have been reported as cholesterol transport pathways other than NPC1L1 [21-23]. Therefore, the expression of these transporters at the mRNA level in MDCK cells was investigated by RT-PCR. Endogenous NPC1L1 and SR-BI expression was confirmed, while expression of endogenous CD36 was not observed (Fig. 2A), and an inhibition assay using the SR-BI inhibitor BLT-1 was therefore performed. However, the uptake of cholesterol was not decreased in the presence of BLT-1 (Fig. 2B). On the other hand, the uptake of cholesterol was decreased in the co-presence of ezetimibe and SR-BI (Fig. 2B). In addition, the inhibition rate was almost the same as that in the presence of ezetimibe only.

In order to clarify the temperature dependency of cholesterol uptake into MDCK cells, an uptake assay was performed on ice. Compared with the condition at 37˚C, the uptake of cholesterol was substantially decreased in both mock cells and NPC1L1 cells (Fig. 2C). There was no difference in uptake between mock cells and NPC1L1 cells on ice.

3.4. Expression, localization and transport activity of NPC1L1 in Xenopus laevis oocytes

The expression of NPC1L1 was observed in oocytes injected with NPC1L1 cRNA, while the expression of NPC1L1 was not observed in oocytes injected with DEPC-treated

water (Fig. 3A). Immunohistochemical staining was performed to determine the localization of NPC1L1 on the cell membrane (Fig. 3B-G). In oocytes with injected NPC1L1 cRNA, the expression level of NPC1L1 on the cell membrane increased with increase in the number of days of culture after injection. There was no difference in the expression level when the amount of injected cRNA was increased from 30 ng to 60 ng.

To analyze the function of NPC1L1, a [^{3}H]-cholesterol uptake assay was performed using NPC1L1 cRNA-injected oocytes. The transport activity was increased in NPC1L1 cRNA-injected oocytes (NPC1L1 oocytes) compared with that in DEPC-treated water injected oocytes (control) (Fig. 4A). In addition, the uptake of cholesterol was decreased in the presence of ezetimibe in NPC1L1 oocytes. On the other hand, transport activity of NPC1L1 was not observed in oocytes cultured for 3 days (data not shown). This result correlated with the expression level confirmed by immunohistochemical staining (Fig. 3). Next, the time course of cholesterol uptake in oocytes was observed (Fig. 4B). The uptake of cholesterol was increased in a time-dependent manner, and the uptake was substantially increased in NPC1L1 oocytes compared with that in the control. Furthermore, the inhibitory effect of ezetimibe on NPC1L1-mediated cholesterol uptake in oocytes was examined. In the control, there was no statistically significant decrease regardless of the concentration of ezetimibe (Fig. 4C). On the other hand, in the NPC1L1 oocytes, cholesterol uptake was inhibited in a ezetimibe concentration-dependent manner, and the inhibitory effect reached a

plateau at 25 μM. Compared to no treatment with ezetimibe. In addition, NPC1L1-dependent uptake was not completely inhibited even at an ezetimibe concentration of 50 μM.

3.5. Evaluation of the characteristics of NPC1L1-mediated cholesterol transport using Xenopus laevis oocytes

Finally, the effect of the L216A mutation on cholesterol uptake was evaluated using *Xenopus laevis* oocytes. NPC1L1 L216A cRNA was injected into oocytes, and the expression and localization of NPC1L1 L216A were confirmed by Western blot analysis and immunohistochemical staining, respectively (Fig. 5A, B). The cholesterol uptake of NPC1L1 L216A cRNA-injected oocytes (NPC1L1 L216A oocytes) was decreased compared with that of NPC1L1 cRNA-injected oocytes (Fig. 5C). On the other hand, there was no difference in the cholesterol uptake between control and NPC1L1 L216A oocytes.

4. Discussion

In this study, the characteristics of micellar cholesterol transport via NPC1L1 were evaluated using a *Xenopus laevis* oocyte expression system in addition to a conventional cell

line with stable expression. First, the particle size distributions of the prepared emulsions and mixed micelles for a cholesterol uptake assay were analyzed. The emulsion was composed of isopropyl myristate as the oil component and sodium taurocholate, the main component of bile acid, as the surfactant, and the average particle size was calculated to be 206.7 ± 2.82 nm (Table 2). On the other hand, the mixed micelle was composed of sodium taurocholate, phosphatidylcholine, and cholesterol, and the average particle size was calculated to be 12.83 ± 1.65 nm. It was previously reported that mixed micelles composed of bile salts and phospholipids were spherical particles with a particle size of about 10 nm [24], and the mixed micelles prepared in this study are considered to be similar to those prepared in the previous study.

The results of RT-PCR, Western blot analysis, and uptake assays showed that endogenous NPC1L1 is expressed and functions in MDCK cells (Fig. 1A-D). These results support the results of a previous study showing endogenous NPC1L1 transport activity in MDCKII cells, one of the cell lines isolated from MDCK cells with high passage numbers [14]. Using NPC1L1 cells, emulsified and micellar cholesterol uptake assays were performed, but these formulations showed different recognition by NPC1L1 (Fig. 1C, D). In a previous study, it was found that recognition of cholesterol by NPC1L1 was increased as the particle size decreased [25], and the results of that study support our results. A possible reason for improvement of the recognition of cholesterol by NPC1L1 is that the smaller particle size

may make it easier for particles to access the brush border membrane of epithelial cells. In other words, it is thought that lipid hydrolases such as lipase improve the bioavailability of cholesterol by miniaturizing the particle size.

In a cholesterol uptake assay using MDCK cells, high endogenous transport activity of cholesterol makes it difficult to evaluate the difference between mock and NPC1L1 cells (Fig. 1E). Here, SR-BI was assumed to be an endogenous cholesterol transport pathway other than NPC1L1 (Fig. 2A). However, the results of an inhibition assay using BLT-1 indicated that SR-BI is not involved in the uptake of micellar cholesterol into MDCK cells (Fig. 2B). SR-BI was previously reported to be involved in the intestinal absorption of various fat-soluble substances such as cholesterol, vitamin E and lycopene [22,26,27]. In this study, the expression level of SR-BI was not quantified, and the low expression level of SR-BI in MDCK cells may make it impossible to evaluate cholesterol uptake via SR-BI.

Moreover, cholesterol uptake was substantially decreased in mock cells on ice, indicating the presence of temperature-dependent endogenous cholesterol transport activity in MDCK cells (Fig. 2C). NPC1L1 cells also showed a remarkable decrease in cholesterol transport activity on ice. There was no difference in the uptake of cholesterol between mock cells and NPC1L1 cells on ice. There results suggest that the transport activity of NPC1L1 almost disappears in MDCK cells on ice. On the other hand, cholesterol uptake, though at a low level, was observed in MDCK cells on ice, indicating that some transport pathways other

than NPC1L1 are involved in the uptake of cholesterol into cells. Since SR-BI was shown not to be involved in the uptake of micellar cholesterol uptake, passive diffusion is assumed to be a possible transport pathway. Mixed micelles have a large molecular size and are thought to be difficult to be taken into cells by passive diffusion as they are, but cholesterol in micelles and that in the intermicellar aqueous phase were in equilibrium, and previous reports suggested that monomer cholesterol in the intermicellar aqueous phase was taken into cells by passive diffusion [28,29].

Although the cholesterol transport activity is decreased in the NPC1L1 L216A mutant, MDCK cells may not be the optimal system for evaluation of the characteristics of NPC1L1-mediated cholesterol transport because of the expression of endogenous NPC1L1 (Fig. 1F, G). Hence, a *Xenopus laevis* oocyte expression system was established as a new evaluation system. Expression of NPC1L1 was observed on the cell membranes of NPC1L1 oocytes cultured for 5-6 days (Fig. 3). The estimated molecular weight of NPC1L1 is 145000, but it has a large number of N-glycosylation sites and the apparent molecular weight is known to be 175000 [30]. However, it was not clarified in this study whether glycosylation to NPC1L1 occurs correctly, although the results of a previous study suggested that protein glycosylation was normal in oocytes [31]. Moreover, it was reported that translocation of NPC1L1 between the plasma membrane and endocytic recycling compartment was dependent on the cholesterol concentration [32], and further studies are needed to determine whether

such a cholesterol-dependent subcellular localization change occurs in the oocyte expression system.

In addition, the results of the uptake assay showed that endogenous NPC1L1 does not function in oocytes (Fig. 4). Therefore, it is thought that the oocyte expression system is useful for evaluating the characteristics of NPC1L1-mediated cholesterol uptake. The results of the inhibition assay using oocytes suggested that ezetimibe partially inhibits NPC1L1-mediated cholesterol uptake (Fig. 4C). Hence, the contribution of NPC1L1 to the uptake of cholesterol into MDCK cells observed in the assay for which results are shown in Fig. 1D may be greater than that inhibited by ezetimibe. Furthermore, the lack of endogenous NPC1L1 expression suggests that the oocyte expression system is suitable for mutant analysis, and the effect of L216A mutation on cholesterol uptake was therefore examined using NPC1L1 L216A oocytes. Although NPC1L1 L216A was expressed on the cell membrane as well in the wild type, the cholesterol uptake of NPC1L1 L216A oocytes was substantially decreased compared with that of NPC1L1 oocytes (Fig. 5). These results indicate that leucine at position 216 of NPC1L1 plays an important role in cholesterol uptake as previously reported [16] and that the oocyte expression system can be applied to mutant analysis. Leucine at position 216 of NPC1L1 is located around the cholesterol binding pocket, but its detailed function has not been elucidated. The structure of the cholesterol binding pocket of NPC1L1 has a closed conformation, suggesting the necessity to change to an open

conformation for cholesterol to bind as previously reported [33], and leucine at position 216 of NPC1L1 may be involved in this conformation change. Kinetic analysis of the effects of amino acid mutations around the binding pocket (*e.g.*, L216A) on cholesterol affinity using the oocyte expression system is needed to elucidate the detailed molecular mechanism of NPC1L1-mediated cholesterol transport.

This work is the first study in which the characteristics of NPC1L1-mediated cholesterol transport were evaluated using *Xenopus laevis* oocytes. A conventional cell line with stable expression expresses endogenous NPC1L1, while the *Xenopus laevis* oocyte expression system lacks the expression of endogenous NPC1L1 and has low endogenous cholesterol transport activity. Therefore, the oocyte expression system is useful for evaluating the transport activity of NPC1L1 alone and can also be applied to mutant analysis. The use of the oocyte expression system is expected to lead to elucidation of the detailed molecular mechanism of NPC1L1-mediated cholesterol transport.

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Authorship contributions

Conceived and designed the experiments: SN, YT, MS, YS

Performed the experiments: SN, SY, NT, MN

Analyzed the data: SN, SY, NT

Wrote or contributed to the writing of the manuscript: SN, YT, MS, YS

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Figure legends

Fig. 1. Evaluation of the characteristics of NPC1L1-mediated cholesterol transport using NPC1L1-overexpressed MDCK cells The expression of NPC1L1 was confirmed by RT-PCR (A) and Western blot analysis (B). The transport activity of NPC1L1 was confirmed by a [^{3}H]-cholesterol uptake assay. Cells were incubated in transport buffer containing 2 μM cholesterol, 0.5 mM sodium taurocholate, 20 nM [^{3}H]-cholesterol (1 μCi/mL) and 1% (v/v) isopropyl myristate with or without 50 μM ezetimibe (C) or in transport buffer containing 10 mM sodium taurocholate, 3.33 mM phosphatidylcholine, 2 μM cholesterol and 20 nM [^{3}H]-cholesterol (1 μCi/mL) with or without 50 μM ezetimibe (D) for 30 min at 37˚C. The radioactivity was measured by a liquid scintillation counter to determine the uptake of cholesterol. The time course of the uptake of micellar cholesterol was also examined for the indicated times (E). NPC1L1 L216A cells were prepared, and the effect of L216A mutation on cholesterol uptake was investigated. The expression of NPC1L1 L216A was confirmed by Western blot analysis (F). The transport activity of [^{3}H]-cholesterol in NPC1L1 L216A cells was compared with that in NPC1L1 cells (G). Each point and vertical bar represents the mean ± S.D. of 3 measurements. The means in groups were compared by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test (C, D, G; *$p<0.05$, **$p<0.01$). The means in mock and NPC1L1 cells were compared by the unpaired two-tailed

t-test (E; *p<0.05).

Fig. 2. Contribution of transport pathways other than NPC1L1 to cellular uptake of cholesterol Expression of endogenous NPC1L1, SR-BI, CD36, GAPDH was confirmed by RT-PCR (A). Cells were incubated in a transport buffer containing 10 mM sodium taurocholate, 3.33 mM phosphatidylcholine, 2 μM cholesterol and 20 nM [^{3}H]-cholesterol (1 μCi/mL) with or without 50 μM ezetimibe and 20 μM BLT-1 for 30 min at 37˚C (B). The radioactivity was measured by a liquid scintillation counter to determine the uptake of cholesterol. In addition, to clarify the temperature dependency of cholesterol uptake into MDCK cells, an uptake assay was performed on ice (C). Each vertical bar represents the mean ± S.D. of 3 measurements. The means in groups were compared by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test (**p<0.01).

Fig. 3. Expression and localization of NPC1L1 in *Xenopus laevis* oocytes The expression of NPC1L1 was examined by Western blot analysis (A). Furthermore, the localization of NPC1L1 on the cell membrane was confirmed by immunohistochemical staining. Oocytes injected with DEPC-treated water (B) or 30 ng NPC1L1 cRNA (C, E, F, G) or 60 ng NPC1L1 cRNA (D) were cultured for 3-6 days. Oocytes were probed a with rabbit anti-NPC1L1 antibody and visualized by using a confocal laser scanning microscope. Scale bar represents

0.2 mm in length. (C, D): cultured for 3 days, (E): cultured for 4 days, (F): cultured for 5 days, (G): cultured for 6 days

Fig. 4. NPC1L1-mediated cholesterol uptake and inhibitory effect of ezetimibe in *Xenopus laevis* oocytes The transport activity of NPC1L1 was confirmed by a [^{3}H]-cholesterol uptake assay (A). Oocytes were incubated in ND96 buffer containing 5 mM sodium taurocholate, 1.67 mM phosphatidylcholine, 1 μM cholesterol and 30 nM [^{3}H]-cholesterol (1.5 μCi/mL) with or without 25 μM ezetimibe for 60 min at 25˚C. The radioactivity was measured by a liquid scintillation counter to determine the uptake of cholesterol. The time course of the uptake of micellar cholesterol was also examined for the indicated times (B). The inhibitory effect of ezetimibe on NPC1L1-mediated cholesterol uptake was examined by incubating ezetimibe at the indicated concentrations. Each point and vertical bar represents the mean ± S.D. of 3-6 measurements. The means in groups were compared by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test (A, C; *$p<0.05$, **$p<0.01$). The means in control and NPC1L1 oocytes were compared by the unpaired two-tailed t-test (B; *$p<0.05$, **$p<0.01$).

Fig. 5. Evaluation of the characteristics of NPC1L1-mediated cholesterol transport using *Xenopus laevis* oocytes The expression and localization of NPC1L1 L216A were examined

by Western blot analysis (A) and immunohistochemical staining (B), respectively. Scale bar represents 0.2 mm in length. The effect of L216A mutation on the transport activity of NPC1L1 was examined by a [^{3}H]-cholesterol uptake assay (C). Oocytes were incubated in ND96 buffer containing 5 mM sodium taurocholate, 1.67 mM phosphatidylcholine, 1 μM cholesterol and 30 nM [^{3}H]-cholesterol (1.5 μCi/mL) with or without 25 μM ezetimibe for 60 min at 25˚C. The radioactivity was measured by a liquid scintillation counter to determine the uptake of cholesterol. Each vertical bar represents the mean with S.D. of 4-5 measurements. The means in groups were compared by two-way factorial ANOVA followed by Tukey-Kramer's multiple comparisons test (**$p<0.01$).

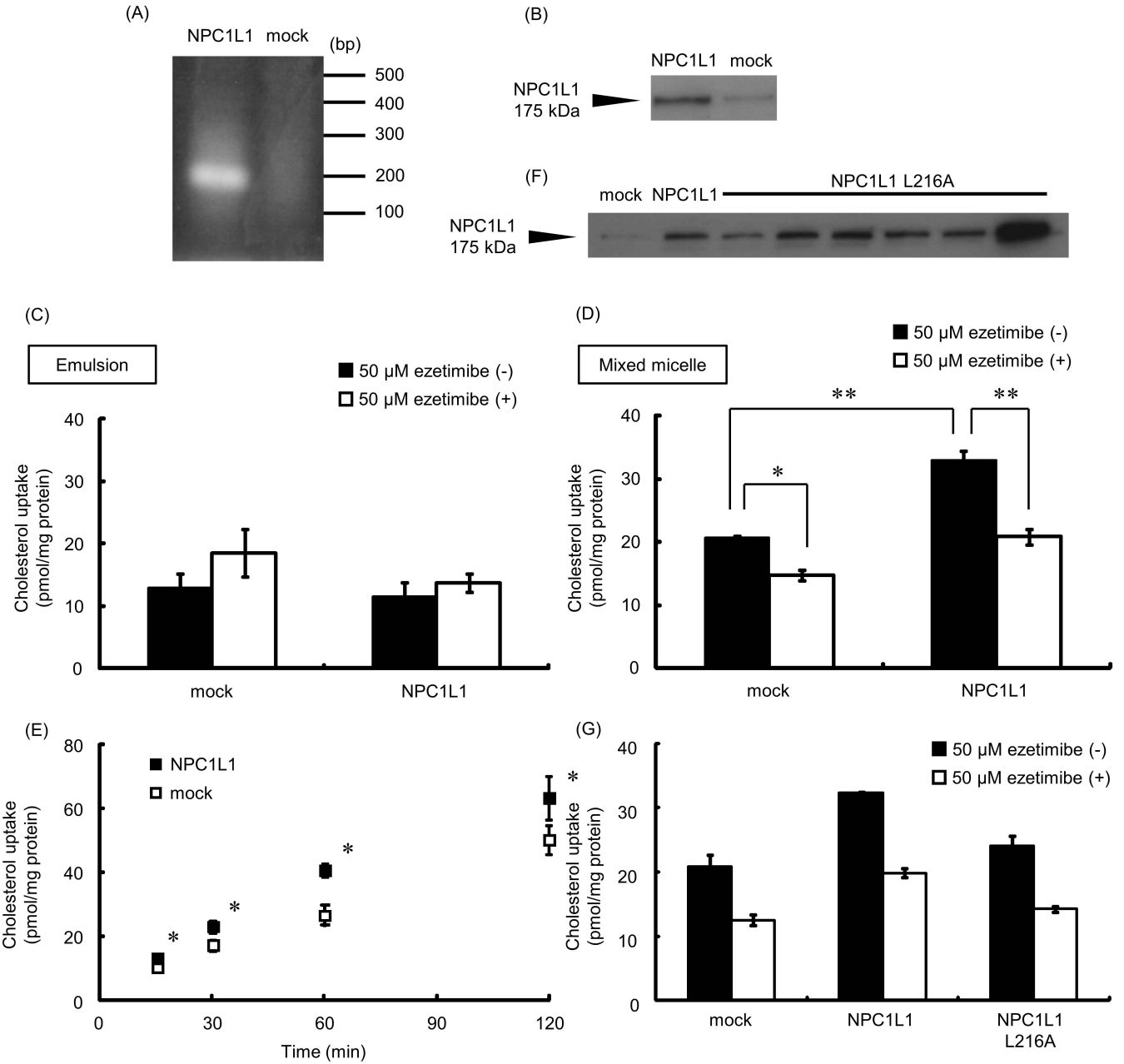

(A)
NPC1L1
mock
(bp)
500
400
300
200
100
(B)
NPC1L1
mock
NPC1L1
175 kDa
(F)
mock
NPC1L1
NPC1L1 L216A
NPC1L1
175 kDa
(C)
Emulsion
50 μM ezetimibe (-)
50 μM ezetimibe (+)
Cholesterol uptake
(pmol/mg protein)
mock
NPC1L1
(D)
Mixed micelle
50 μM ezetimibe (-)
50 μM ezetimibe (+)
**
**
*
Cholesterol uptake
(pmol/mg protein)
mock
NPC1L1
(E)
NPC1L1
mock
Cholesterol uptake
(pmol/mg protein)
Time (min)
(G)
50 μM ezetimibe (-)
50 μM ezetimibe (+)
Cholesterol uptake
(pmol/mg protein)
mock
NPC1L1
NPC1L1
L216A

Fig. 1

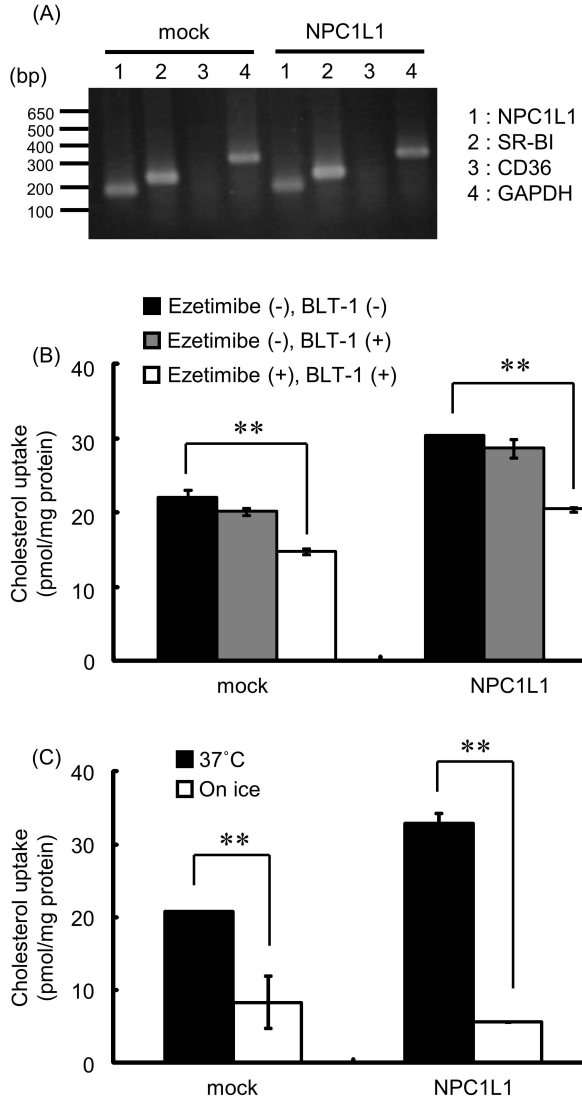

(A)
mock
NPC1L1
(bp)
1 2 3 4 1 2 3 4
650
500
400
300
200
100
1 : NPC1L1
2 : SR-BI
3 : CD36
4 : GAPDH
Ezetimibe (-), BLT-1 (-)
Ezetimibe (-), BLT-1 (+)
Ezetimibe (+), BLT-1 (+)
(B)
Cholesterol uptake (pmol/mg protein)
40
30
20
10
0
**
**
mock
NPC1L1
(C)
37˚C
On ice
Cholesterol uptake (pmol/mg protein)
40
30
20
10
0
**
**
mock
NPC1L1

Fig. 2

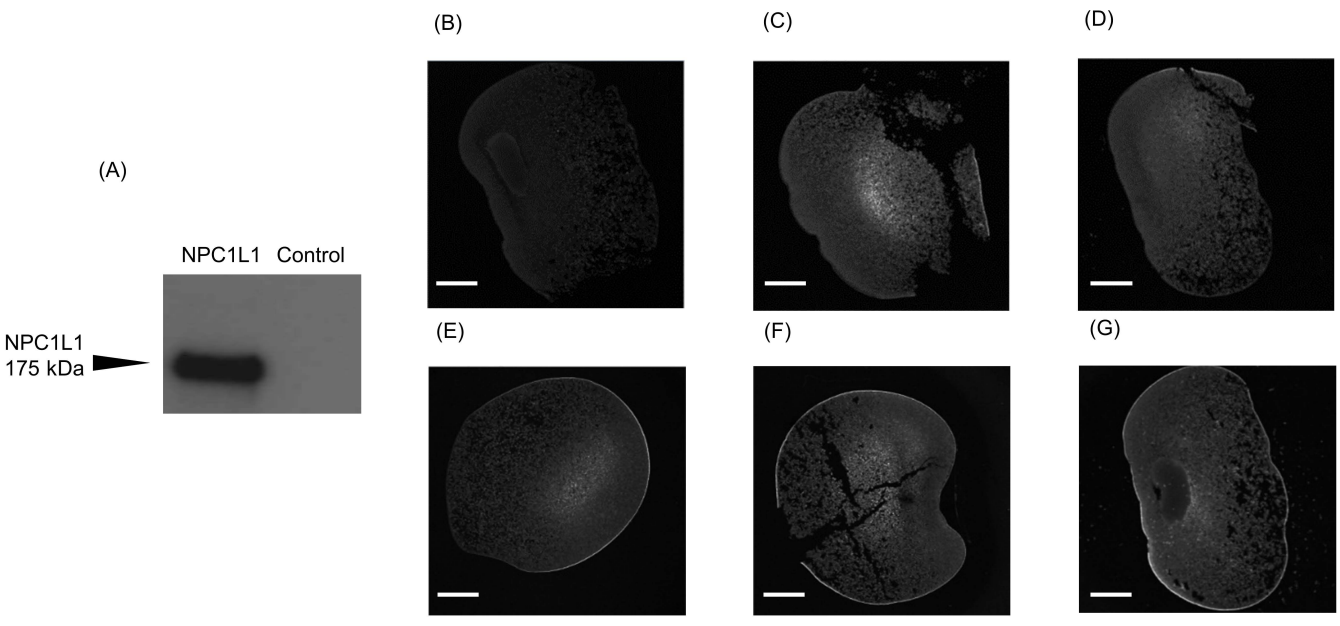


Fig. 3

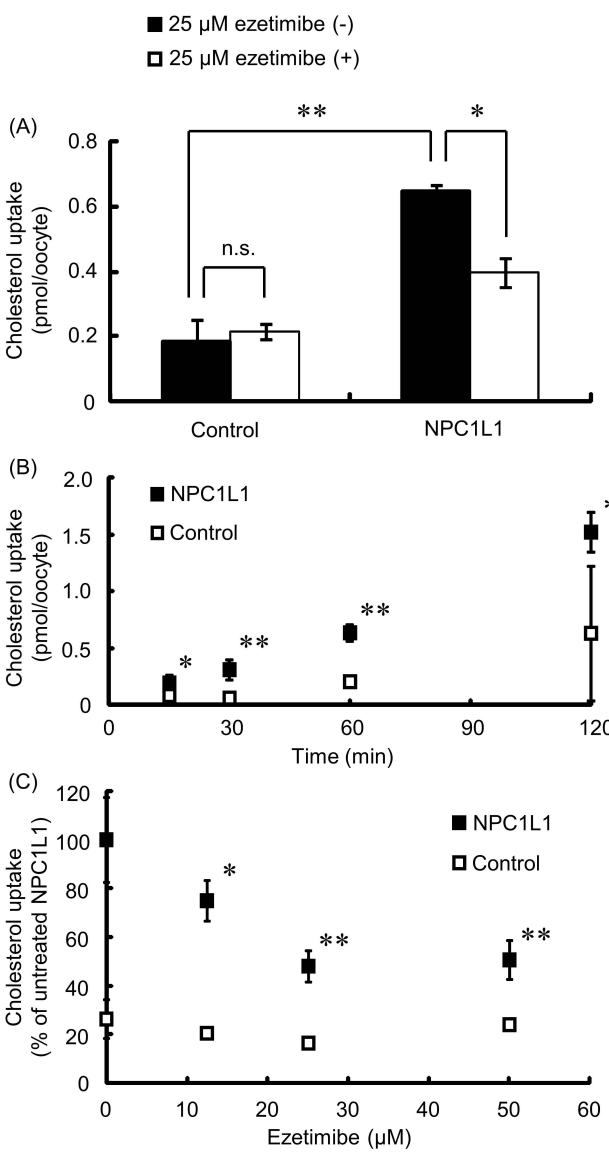

25 μM ezetimibe (-)
25 μM ezetimibe (+)
(A)
Cholesterol uptake (pmol/oocyte)
0.8
0.6
0.4
0.2
0
**
*
n.s.
Control
NPC1L1
(B)
Cholesterol uptake (pmol/oocyte)
2.0
1.5
1.0
0.5
0
NPC1L1
Control
*
**
**
*
0
30
60
90
120
Time (min)
(C)
Cholesterol uptake (% of untreated NPC1L1)
120
100
80
60
40
20
0
NPC1L1
Control
*
**
**
0
10
20
30
40
50
60
Ezetimibe (μM)

Fig. 4

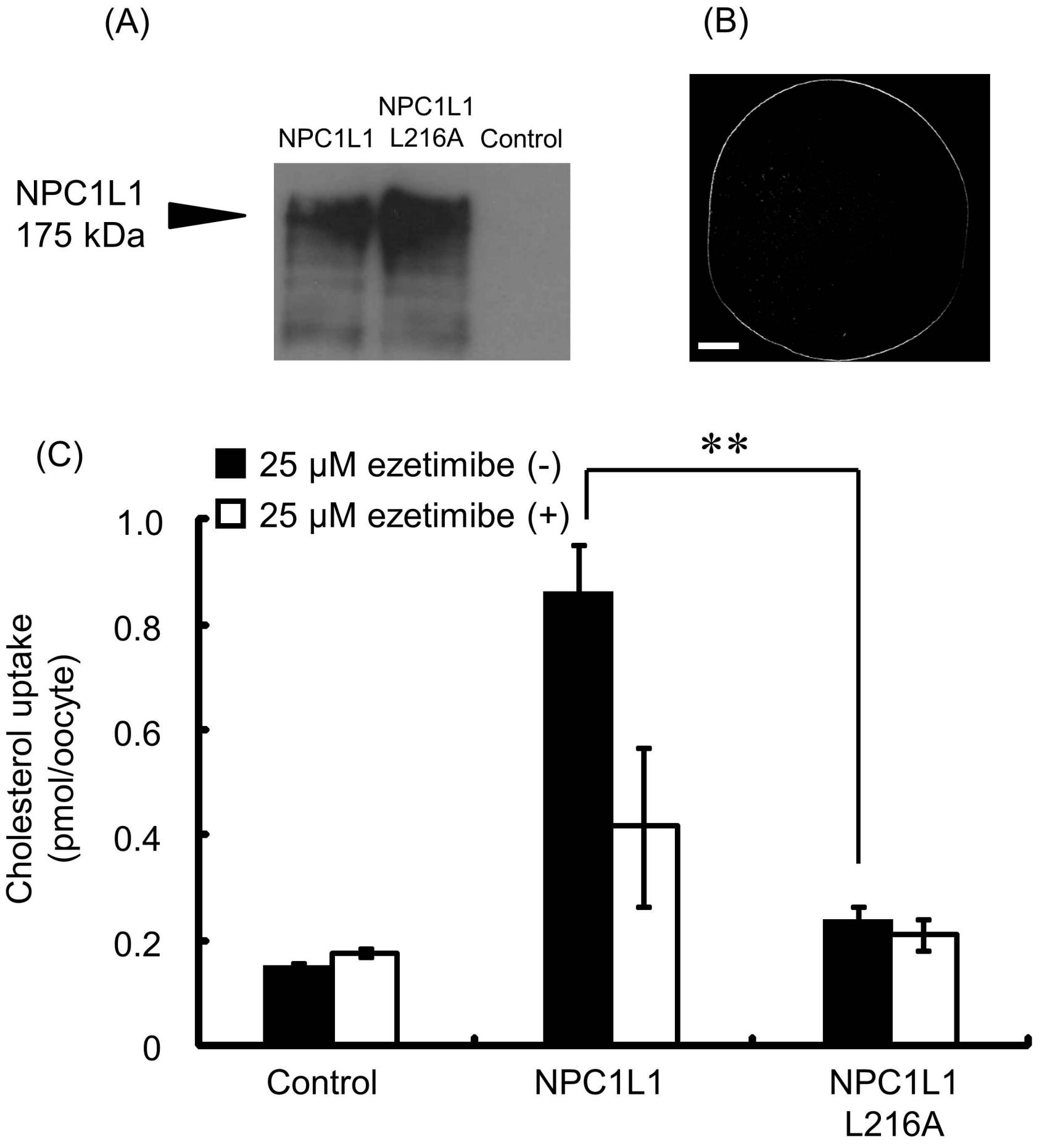

(A)
NPC1L1
NPC1L1 L216A Control
NPC1L1
175 kDa
(B)
(C)
25 μM ezetimibe (-)
25 μM ezetimibe (+)
**
Cholesterol uptake
(pmol/oocyte)
1.0
0.8
0.6
0.4
0.2
0
Control
NPC1L1
NPC1L1
L216A

Fig. 5

Table 1 Primer sequences used for RT-PCR analysis

Primer name	Primer sequence	Product size
human NPC1L1 Forward	5’- ATGAGTCCCAAGGTGACGAC -3’	166 bp
human NPC1L1 Reverse	5’- GTGACCACAGCGAAGACAGA -3’	
dog NPC1L1 Forward	5’- AACGAGTCCCAGGGCAATG -3’	167 bp
dog NPC1L1 Reverse	5’- GTAAGCAGGGCAAAAGCAGA -3’	
dog SR-BI Forward	5’- CCATAGGCGAGATCATGTGG -3’	216 bp
dog SR-BI Reverse	5’- GCCAGAAGTTGACCTTGCTG -3’	
dog CD36 Forward	5’- GAGACATGCTGATTGAGAAGACA -3’	552 bp
dog CD36 Reverse	5’- CTGAATAAACTCCATCGACAGTG -3’	
dog GAPDH Forward	5’- CCAGTATGATTCTACCCACGG -3’	306 bp
dog GAPDH Reverse	5’- TTGCTGACAATCTTGAGGGAG -3’	

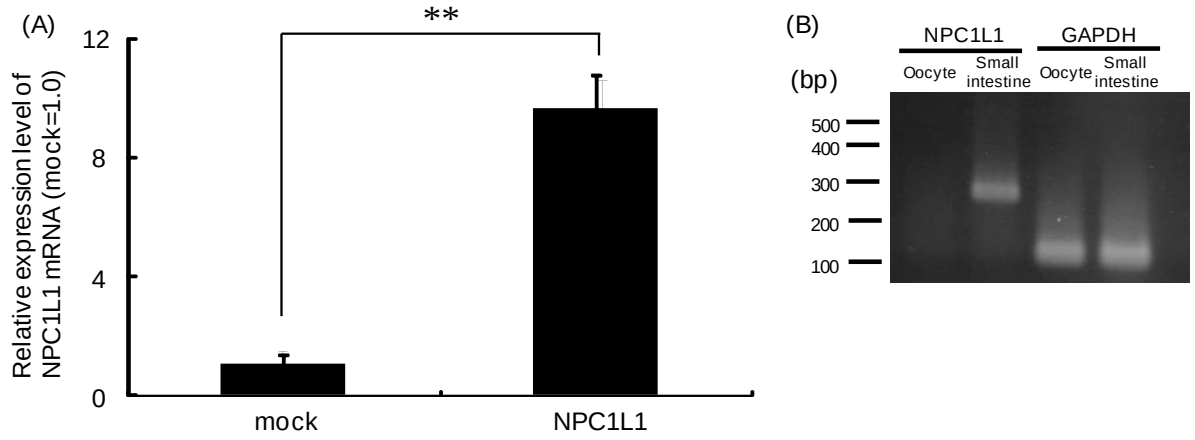
Table 2 Average particle size (nm) and PdI value of each formulation

	Particle size	PdI
Emulsion dispersed in transport buffer	206.7 ± 2.82	0.18 ± 0.022
Mixed micelle dispersed in transport buffer	12.83 ± 1.65	0.37 ± 0.031
Mixed micelle dispersed in ND96 buffer	14.17 ± 1.52	0.39 ± 0.057

Average particle size and PdI value were determined by using a quasi-elastic light scattering method. Each parameter represents the mean ± S.D. of 3-5 measurements.

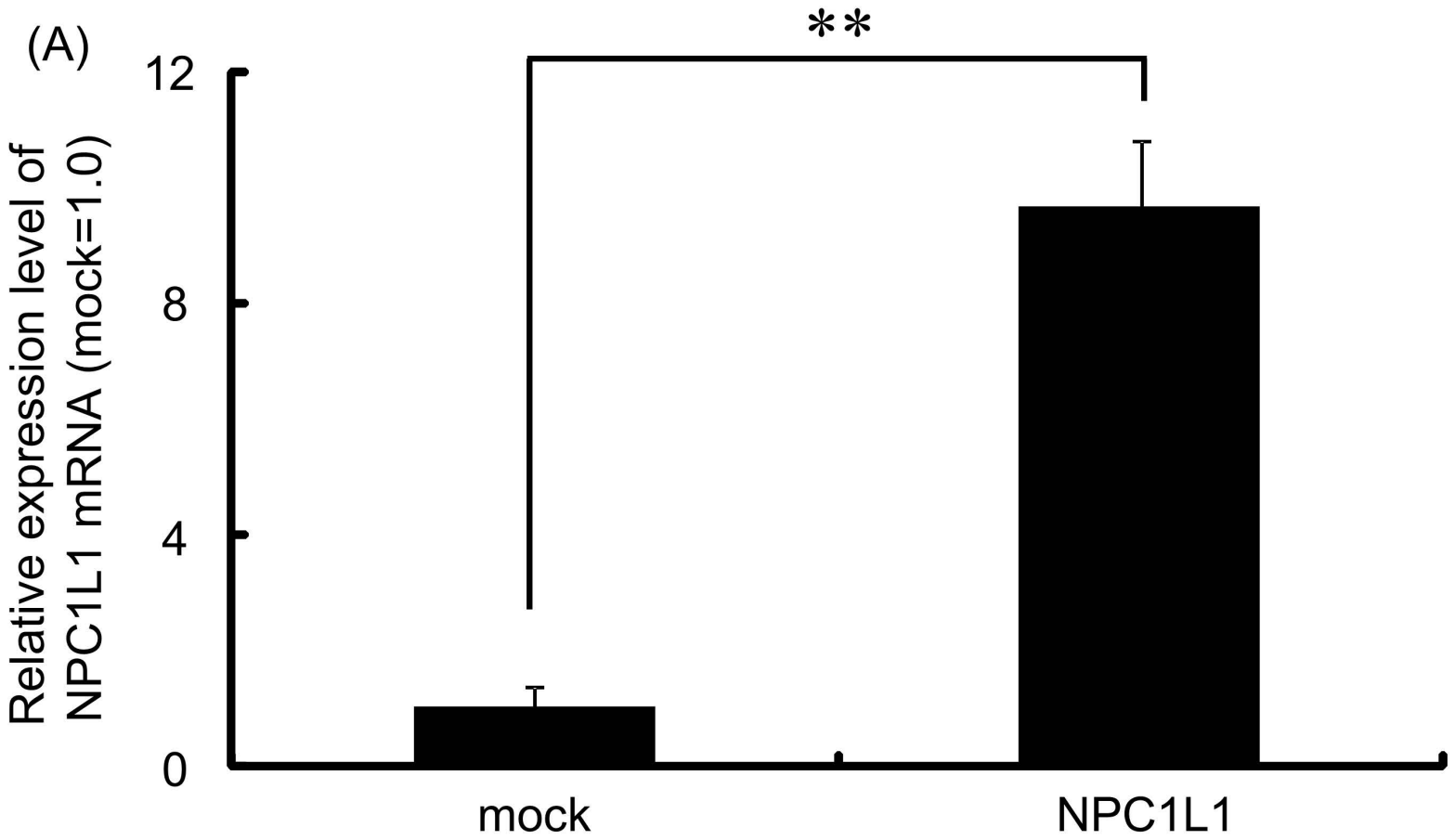
Supplementary Table 1 Primer sequences used for RT-PCR and real-time PCR analysis.

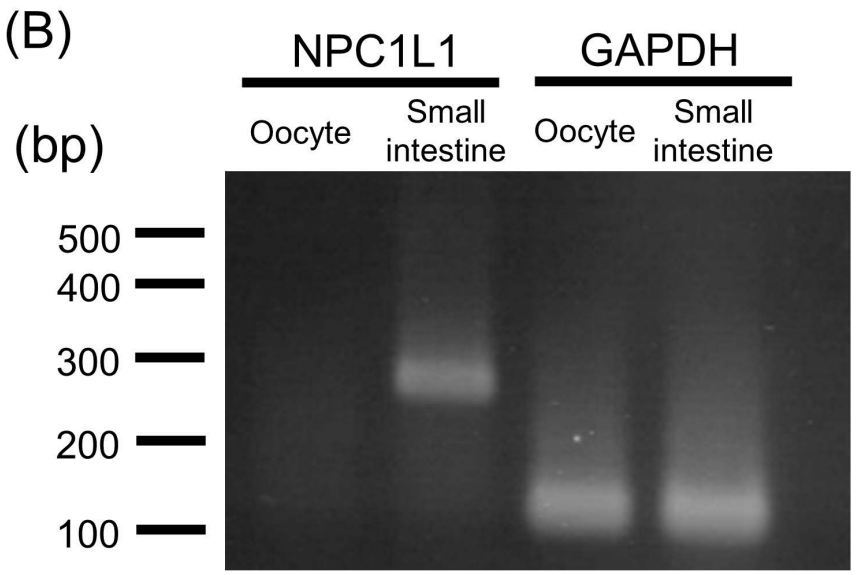
Primer name	Primer sequence	Product size
NPC1L1 for real-time PCR Forward	5′- GGCTACAACTTCTCCAGCGA -3′	192 bp
NPC1L1 for real-time PCR Reverse	5′- ATAAAGGCGGCAGCAGGA -3′	
GAPDH for real-time PCR Forward	5′- GGCATCCTGGGCTACACTG -3′	122 bp
GAPDH for real-time PCR Reverse	5′- GAAATGAGCTTGACAAAGTGGTC -3′	
Xenopus NPC1L1 Forward	5′- CAACAGCCAAGCAAGACAAG -3′	270 bp
Xenopus NPC1L1 Reverse	5′- TTCAGGGGAGCATAGCAGA -3′	
Xenopus GAPDH Forward	5′- CACTGCCACCCAGAAGAC -3′	135 bp
Xenopus GAPDH Reverse	5′- CCGTTCAGCTCAGGGAT -3′	



Supplementary Fig. 1. Expression of endogenous NPC1L1 in MDCK cells and oocyte

Relative expression level of NPC1L1 in MDCK cells was measured by real-time PCR using primers that bind to both dog and human NPC1L1 sequences (A). GAPDH was selected as the internal control gene. Each bar represents the mean ± S.D. of 3 measurements (** $p < 0.01$ by the unpaired two-tailed t-test). On the other hand, expression of endogenous NPC1L1 in oocytes and small intestine of *Xenopus laevis* frogs was confirmed by RT-PCR (B). The expression of GAPDH was also examined as a positive control.





Supplemental Fig. 1