

Research Paper

HNF4 α is a Crucial Modulator of the Cholesterol-Dependent Regulation of NPC1L1

Yuki Iwayanagi,¹ Tappei Takada,^{1,2} and Hiroshi Suzuki¹

Received July 30, 2007; accepted November 6, 2007; published online December 14, 2007

Purpose. Niemann-Pick C1-like 1 (NPC1L1) has been identified as a target of ezetimibe and found to be responsible for intestinal cholesterol absorption. Although, it was recently demonstrated that sterol responsive element binding protein 2 (SREBP2) is responsible for the cholesterol-dependent down-regulation of NPC1L1, the molecular mechanism of NPC1L1 expression is not fully understood. In the present study, we examined the involvement of hepatocyte nuclear factor 4 α (HNF4 α), a key modulator of lipid metabolism, in the transcriptional regulation of human NPC1L1 gene.

Methods. Reporter gene assays and EMSAs were performed using human NPC1L1 promoter constructs and the effect of siHNF4 α was examined.

Results. Transfection of SREBP2 induced the transcriptional activities of NPC1L1 and additional transfection of HNF4 α results in a marked stimulation of the activities. Studies with deletion mutants indicated that important elements are located within 264 nt upstream in the human NPC1L1 promoter. In addition, studies with mutations in putative binding sites of HNF4 α indicated the existence of binding sites in -209 to -197 and -52 to -40. Moreover, HNF4 α knockdown resulted in the reduced expression and regulation by cholesterol.

Conclusions. It is concluded that HNF4 α plays a crucial role in the expression and regulation of human NPC1L1 gene.

KEY WORDS: cholesterol; HNF4 α ; intestinal transport; NPC1L1; transcriptional regulation.

INTRODUCTION

Recently, a novel cholesterol lowering drug, ezetimibe, has been used to treat hypercholesterolemia (1,2). Ezetimibe is believed to act by inhibiting intestinal cholesterol absorption (1). A putative transporter protein on the membrane, Niemann-Pick C1-like 1 (NPC1L1), was identified as a molecular target of ezetimibe (3). Pharmacological studies with ezetimibe suggested that NPC1L1 may play an important role in cholesterol absorption in the intestine (4). Indeed, the NPC1L1 null mice showed significant reduction in intestinal cholesterol absorption and the residual cholesterol

absorption was insensitive to ezetimibe treatment (5). In addition, a paper published very recently showed that NPC1L1 contributes to cholesterol reuptake from bile (6).

Previously, it was reported that the intake of cholesterol results in the suppression of NPC1L1 expression in mouse intestine (5). This result appears to be quite reasonable taking physiological function of NPC1L1 into consideration. Concerning the mechanism of regulation of expression, some reports have been published which demonstrate the involvement of transcriptional factors in cholesterol-dependent regulation of NPC1L1 (7,8). For instance, liver X receptor (LXR) is reported to be involved in down-regulation of NPC1L1 expression in a human enterocyte cell line (Caco-2/TC7) (7). In addition, NPC1L1 mRNA levels were reduced in duodenum of mice treated with an LXR agonist (7). However, the detailed mechanism of the LXR-dependent regulation of NPC1L1 transcription remains to be clarified.

In addition to LXR, sterol regulatory element binding protein 2 (SREBP2) was recently reported to activate NPC1L1 transcription and mediates the down-regulation of NPC1L1 by cholesterol in Caco-2 cells (9). The authors found two sterol responsive elements (SRE) in the 5'-flanking region of human NPC1L1. SREBP2 is a widely known transcriptional factor which maintains the homeostasis of cholesterol in the whole body, and its activation is negatively regulated by sterols. Moreover, based on the sequence in the 5'-flanking region of human NPC1L1, the presence of putative binding elements for hepatocyte nuclear factor 4 α

Yuki Iwayanagi and Tappei Takada are equally contributed.

¹ Department of Pharmacy, The University of Tokyo Hospital, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan.

² To whom correspondence should be addressed. (e-mail: tappei-tky@umin.ac.jp)

ABBREVIATIONS: DMEM, Dulbecco's Modified Eagle Medium; DR, direct repeat; EMSA, electrophoretic mobility shift assay; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; HNF4 α , hepatocyte nuclear factor 4 α ; LDLR, low density lipoprotein receptor; LXR, liver X receptor; NF-Y, nuclear factor-Y; NPC1L1, Niemann-Pick C1-like 1; 25-HCH, 25-hydroxy cholesterol; PGC-1 α , peroxisome proliferator-activated receptor (PPAR)- γ coactivator-1 α ; SRE, sterol responsive element; SREBP, sterol responsive element binding protein.

(HNF4 α) has been assumed (between -848 and -841, between -670 and -652, and between -617 and -601) (10,11). HNF4 α is an orphan member of the nuclear receptor superfamily which is expressed in the liver, intestine, kidney and pancreas and known to be a key modulator of lipid and glucose metabolism. It is also reported to play important roles in the expression of drug metabolizing enzymes and drug transporters in human liver (12,13) and kidney (14). Additionally, HNF4 α is reported to interact with SREBP2 in regulating genes related to cholesterol metabolism, such as low density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (15). Mice lacking hepatic HNF4 α expression exhibit hepatic accumulation of lipids and greatly reduced serum cholesterol and triglyceride levels (16). Based on these findings, we investigated whether HNF4 α transactivates NPC1L1 and is involved in the SREBP2-mediated regulation.

MATERIALS AND METHODS

Materials. Restriction enzymes, 25-hydroxycholesterol (25-HCH) and Premix Taq were purchased from TAKARA BIO INC (Siga, Japan). pGEM-T Easy Vector Systems, Dual-Luciferase Reporter Assay System, pGL3-basic and pRL-SV40 vectors were purchased from Promega KK (Tokyo, Japan). pcDNA3.1(+) vector was purchased from Invitrogen (Carlsbad, CA). Quick change mutagenesis kits were purchased from Stratagene (La Jolla, CA). Phenol red-free Dulbecco's Modified Eagle Medium (DMEM) and penicillin-streptomycin were purchased from GIBCO (Tokyo, Japan). FUGENE6 and lovastatin were purchased from Roche Diagnostics (Tokyo, Japan). Cholesterol was purchased from Wako (Osaka, Japan).

Cloning of the 5'-Flanking Region of Human NPC1L1 and Vector Construction. Based on the human NPC1L1 cDNA sequence, we have identified the 5'-flanking sequence of NPC1L1 (GenBank accession number AC004938) with the BLAST algorithm. Based on the identified sequences of 5'-flanking region, promoter sense primer (5'-gggtcatctcccaataaac-3') and antisense primer (5'-aggcctcaggaacagccaag-3') were prepared. Amplification by these pair of primers using human genome (CORIELL CELL REPOSITORIES, HD07, human variation panel-Japanese) as a template resulted in the generation of an approximately 1.3 kbp fragment of the promoter region of NPC1L1 (from -1,315 to +20). Amplified products were inserted into pGEM-T Easy Vector and the entire sequences were found to be identical with that in GenBank™AC004938. Using this plasmid as a template, additional PCR was performed to subclone the genome sequence between the Kpn I and Hind III sites in pGL3-Basic vector. The resulting plasmids were referred to as p1315/Luc containing the region from -1,315 to +20 of the human NPC1L1 promoters and p264/Luc containing the region from -264 to +20.

Construction of NPC1L1 Promoter Mutant. Two direct repeat-1 (DR-1) elements in the NPC1L1 promoter regions were mutated by the site-directed mutagenesis technique. The following sense and antisense primers were used to construct the two mutated vectors. For the production of

Mut-p-264 (-209/-197)/Luc, in which the sequence between -209 and -197 was mutated, the sense and antisense primers were 5'-tagctgactcCATTACCCTGAACcggctcgttt-3' and 5'-aaagcgaccgGTTTCAGGGTAATGgagtcagcta-3', respectively. For the production of Mut-p-264 (-52/-40)/Luc, in which the sequence between -52 and -40 was mutated, the sense and antisense primers were 5'-taaccagtcCATTACCCTGAACcatcgaagg-3' and 5'-ccctctgatGTTTCAGGGTAATGgactgggta-3', respectively.

Vector Construction for the Expression of Nuclear Receptors. SREBP1a/1c/2 DNAs were amplified by PCR from total RNA of HepG2 cell. The cDNAs encoding the nuclear form of SREBPs were amplified with the EcoRI site attached at the 5'-end, and with the Xba I site attached at the 3'-end by PCR. After insertion of the amplified cDNAs for SREBPs into pGEM-T Easy Vector, the inserted fragments were digested with EcoRI and Xba I, and were ligated into pcDNA3.1(+) vector plasmid. pcDNA3.1(+) containing HNF4 α cDNA was obtained as described previously (17).

Luciferase Assay. HepG2 cells were plated on day 0 at a density of 1.5×10^5 cells/well on 24-well plates and grown in phenol red-free DMEM with 10% charcoal-absorbed fetal bovine serum and 1% penicillin-streptomycin. On day 1, cells were transfected with 500 ng/well of pGL3-Basic vector, with or without NPC1L1 promoter, using FUGENE6 at a DNA/lipid ratio of 1:3. In some experiments, co-transfections were performed with 100 ng/well of SREBPs in pcDNA3.1(+) vector and/or 500 ng/well of HNF4 α in pcDNA3.1(+) vector. All wells were also co-transfected with 50 ng/well of pRL-SV40 vector to correct the transfection efficiency. At 48 h after transfection, luciferase activities were quantified by Luminescencer MCA (ATTO, Tokyo, Japan) using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol. All results were confirmed by additional experiments.

Electrophoretic Mobility Shift Assay (EMSA). EMSAs were performed with *in vitro* translated proteins and a DIG

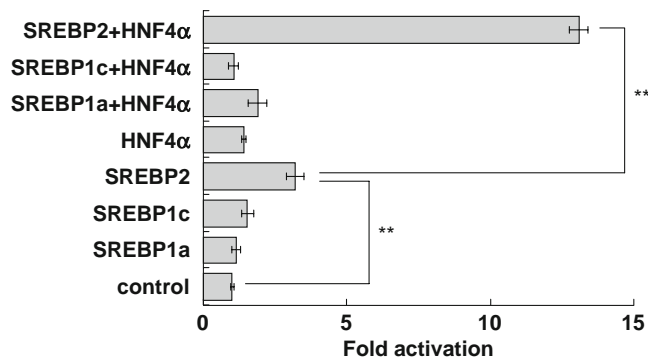


Fig. 1. Activation of reporter-linked human NPC1L1 promoter. HepG2 cells were co-transfected with luciferase-linked human NPC1L1 promoter construct (-1,315 to +20) and expression vectors for SREBP1a, SREBP1c, SREBP2 with or without HNF4 α for 48 h. The fold activation values were calculated by dividing the luciferase activity of each construct by that of control cells transfected with pcDNA3.1(+). Values are expressed as the mean \pm S.E. ($n=3$). Double asterisk significantly different from the control or SREBP2 introduced cells by Student-Newman-Keuls test ($p < 0.01$).

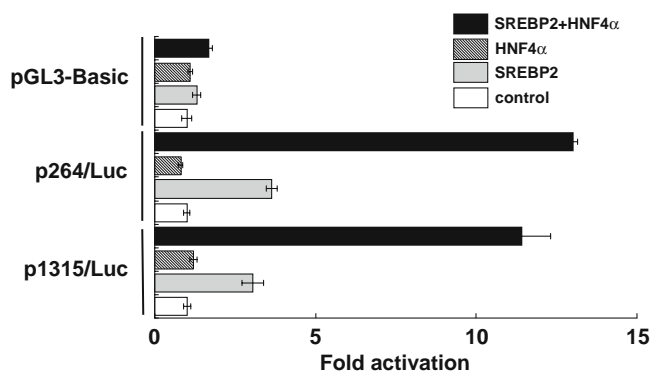
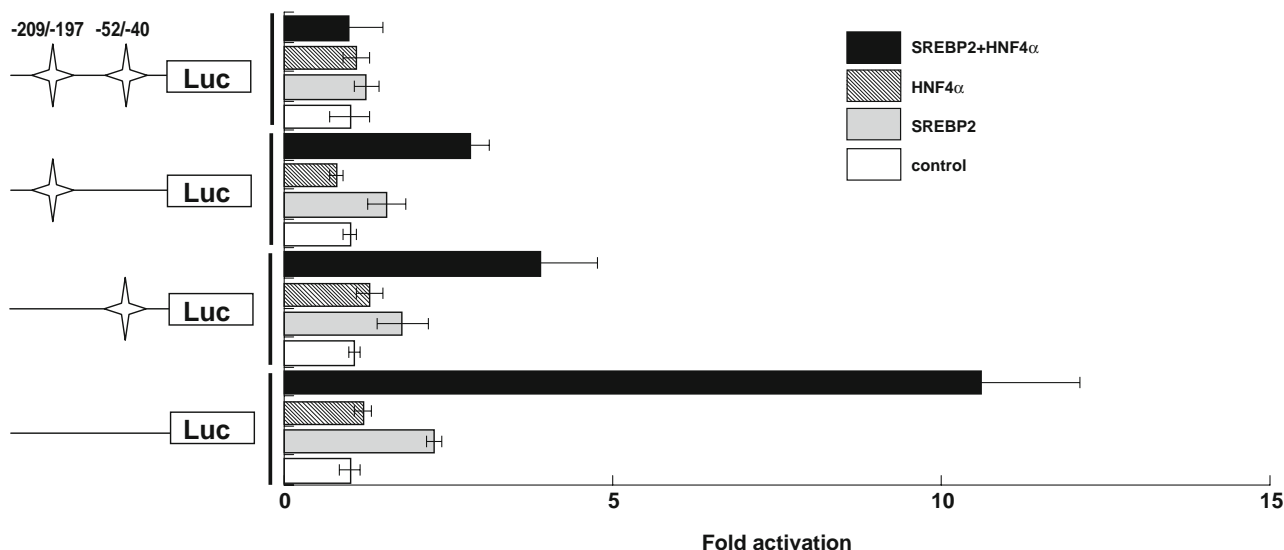


Fig. 2. Activity of human NPC1L1 promoters. HepG2 cells were transfected with deletion mutants of NPC1L1 promoter constructs. The fold activation values were calculated by dividing the activities of each promoter by that of control cells co-transfected only with pcDNA3.1(+) expression vector. Values are expressed as the mean \pm S.E. ($n=3$).

Gel Shift Kit (Roche Diagnostics) according to the manufacturer's protocol. HNF4 α proteins were synthesized using the TNT Quick Coupled Transcription/Translation Systems (Promega KK). The strand sequences for NPC1L1 DR-1 sequences located between -209 and -197 were 5'-tagctgactcagccctctggcttcggtcgcttt-3' and 5'-aaagcggggcctgtggactggggccaaacagtgagggtggtggatcattaaccagcagggctcagcctca-3', and DR-1 sequences located between -52 and -40 were 5'-taaccagtcaggccagggttgcctgaagg-3' and 5'-cccttcgatgacaacctggcctgactgggtta-3', respectively. The mutated NPC1L1 DR-1 sequences (mut NPC1L1) were the same as the sequences used for constructing mutated DR-1 promoters. The double-stranded oligonucleotides were end-labeled with digoxigenin-11-ddUTP and incubated with HNF4 α proteins at 20°C for 2.5 h. The mixture was transferred to a native polyacrylamide gel and electrophoretically separated. Following the separation, the oligonucleotide-protein complexes were transferred onto positively charged nylon membrane by electroblotting. Then, digoxigenin-labeled complexes on the membrane were detected by anti-digoxigenin antibody.



HNF4 α

-212 ctcagccctctggcttcggtcgctttgggaagtgagtgaggaccctagcacctgcgtgatgaggctcatct -142

-141 aaagcggggcctgtggactggggccaaacagtgagggtggtggatcattaaccagcagggctcagcctca -71

-70 ttggtccctaaccagtcaggccagggttgcctgaaggaggctgccttaatgtgtgttcagccC +1

HNF4 α SREBP2

	Putative HNF4 α binding sites	Mutation introduced in
p264/Luc: -209/-197bp	tagctgactcAGCCCTCTGGCTTcggtcgcttt	tagctgactcCATTACCCTGAACcggtcgcttt
p264/Luc: -52/-40bp	taaccagtcAGGCCAGGGTTGTcatcgaagg	taaccagtcCATTACCCTGAACcatcgaagg

Fig. 3. Effect of mutation in putative HNF4 α binding sites on the promoter activity. Mutations were introduced into NPC1L1 promoter region. The first, second, third and fourth columns represent the results of luciferase assays obtained with reporter genes containing mutations between -209 and -197 and between -52 and -40, mutations between -209 and -197, mutations between -52 and -40, and no mutations, respectively. Concerning the production of the mutated sequence between -209 and -197, cattacctgaac was inserted instead of agccctctggctt, whereas cattacctgaac was inserted instead of aggcagggttgc for -52/-40. HepG2 cells were transfected with wild type or a mutant of luciferase-linked NPC1L1 promoter constructs (-264 to +20) in order to perform the luciferase assay. The fold activation values were calculated by dividing the activities of each promoter by that of control cells co-transfected only with pcDNA3.1(+) expression vector. Values are expressed as the mean \pm S.E. ($n=3$).

Transfection of Cells with siHNF4 α and Real-Time Quantitative PCR. For determination of the effect of HNF4 α -knockdown on the expression levels of endogenous NPC1L1, HepG2 cells were suspended in DMEM without antibiotics so that 1 ml contains 100,000 cells. Approximately 20 pmol/well RNAi targeting HNF4 α (catalogue number M-00346-01; Dharmacon, Lafayette, CO) or siCONTROL designed not to interfere with any human genes were diluted in 200 μ l/well DMEM without serum and antibiotics in the well of the tissue culture plate (12-well format). Then, 2 μ l/well Lipofectamin RNAiMAX (Catalogue number 13778-150; Invitrogen) was added to each well containing the diluted RNAi molecules. After incubation for 20 min at room temperature, 1 ml/well of diluted cells were added. After incubation for 24 h, cells were loaded with 50 μ M 25-HCH or 50 μ M lovastatin if indicated. Then, the cells were harvested 72 h after transfection with RNA-solve reagent (Omega Bio-tek, Doraville, GA) and RNA obtained was reverse-transcribed with ReverTra Ace (TOYOBO, Osaka, Japan). Real-time quantitative PCR was performed using 2 \times SYBR GREEN (Stratagene) and Chromo4 (BIORAD, Tokyo, Japan) with 95 $^{\circ}$ C 10 min followed by the 40 cycles at 95 $^{\circ}$ C for 15 s, 50 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s. Primers for human NPC1L1 (sense and antisense primers were 5'-ggatcactggaagcagtc-3' and 5'-aggtagaaggtgagtcgag-3', respectively), and human HNF4 α (sense and antisense primers were 5'-gtgccaacacaatgccactcacctcag-3' and 5'-ggctcccgaggagcctatagg-3', respectively) and β -actin (sense and antisense primers were 5'-ccggaaggaaaactgacagc-3' and 5'-gtggtgtaagctgtagcc-3', respectively) were used.

RESULTS

SREBP2 and HNF4 α Upregulate the Transcription of Human NPC1L1 Gene. A previous study showed that NPC1L1 promoter activity was transactivated by SREBP2 in Caco-2 cells (9). To examine the effect of SREBPs and HNF4 α on NPC1L1 promoter activity, an approximately 1.3 kbp fragment of NPC1L1 promoter was inserted into upstream of the luciferase gene. This construct was transiently transfected into HepG2 cells with SREBP1a/1c/2 in the presence and absence of HNF4 α expression vectors. The luciferase activity of NPC1L1 was induced by co-transfection of SREBP2, whereas HNF4 α did not affect the activity (Fig. 1). However, a further marked increase was observed when HNF4 α was co-transfected with SREBP2 (Fig. 1). These results indicate that HNF4 α stimulates the transcription of NPC1L1 along with SREBP2. Similar effects of HNF4 α and SREBP2 on the promoter activity of NPC1L1 was also observed in Caco-2 cells (data not shown).

Promoter Activities of the Deletion Mutants of NPC1L1. To determine the location of the crucial elements for the HNF4 α response, we constructed reporter plasmids containing deletion mutants of the 5'-flanking regions of the NPC1L1 promoter. The construct was transiently transfected with SREBP2 and HNF4 α (Fig. 2). The 1,315 nt promoter and 264 nt promoter exhibited almost the same response to SREBP2/HNF4 α (Fig. 2). These results suggest that the regulatory elements for HNF4 α and SREBP2 may be located

within 264 nt upstream of the transcriptional initiation site. This result is consistent with the presence of proximal functional SRE between -35 and -26 (9).

Effects of Mutations in the Putative HNF4 α Binding Site on Promoter Activity. In general, HNF4 α binds to DR-1 like element. It was found that several DR-1 like elements are located within 264 nt in the NPC1L1 promoter region. To determine the functional HNF4 α responsive elements, mutations in DR-1 like elements were introduced by site-directed mutagenesis (Fig. 3). HepG2 cells were transfected with NPC1L1 promoter-luciferase reporter constructs containing the wild type or mutated motifs. As shown in Fig. 3, mutations in either DR-1 (between -209 and -197) or DR-1 (between -52 and -40) resulted in a reduction in the response to SREBP2/HNF4 α , whereas mutations in other DR-1 like elements (such as the sequence between -134 and -122 and between -120 and -108) did not affect the response (data not shown). Furthermore, simultaneous mutations in both DR-1 elements (between -209 and -197 and between -52 and -40) abolished the response. These observations suggest that DR-1 located between -209 and -197 and between -52 and -40 regions upstream of NPC1L1 are necessary for HNF4 α -dependent regulation of NPC1L1.

HNF4 α Directly Binds to the DR-1 Motifs in the NPC1L1 Promoter. EMSA was performed to detect the direct binding of HNF4 α to the two putative elements which were shown to be related to the HNF4 α response by luciferase assay (Fig. 4). It was found that the band for the DNA probe was shifted after incubation with HNF4 α synthesized *in vitro* (Fig. 4). Furthermore, the shift was abolished in the presence of unlabeled competitors, but not by mutated DR-1 (Fig. 4). Together with the results of reporter gene assay, the presence of functional HNF4 α binding elements between -209 and -197 and between -52 and -40 in the NPC1L1 promoter was revealed.

Effect of HNF4 α Specific Knock-Down on the Expression of NPC1L1. To examine the effect of HNF4 α on the endogenous expression of NPC1L1, HNF4 α -specific siRNA was transfected into HepG2 cells. Transfection of the HNF4 α -specific siRNA reduced HNF4 α mRNA to 15 \pm 0% compared with cells transfected with nontargeting siRNAs (Fig. 5A). NPC1L1 mRNA expression was reduced to 22 \pm 8% by transfection of HNF4 α -specific siRNA (Fig. 5B). These results indicate that HNF4 α is crucial for the baseline expression of NPC1L1.

Furthermore, in order to examine the importance of the enhancing effect of HNF4 α on SREBP2 activity, we examined the effect of 25-HCH and lovastatin on the endogenous expression of NPC1L1 when cells were transfected with HNF4 α -specific siRNA (Fig. 5C). It was found that the cells treated with HNF4 α -specific siRNA did not show any response to 25-HCH or lovastatin (Fig. 5C). This result suggests that HNF4 α is crucial for the regulation of NPC1L1 expression by cholesterol.

DISCUSSION

In the present study, the analysis using the 5'-flanking region of human NPC1L1 demonstrated that HNF4 α is

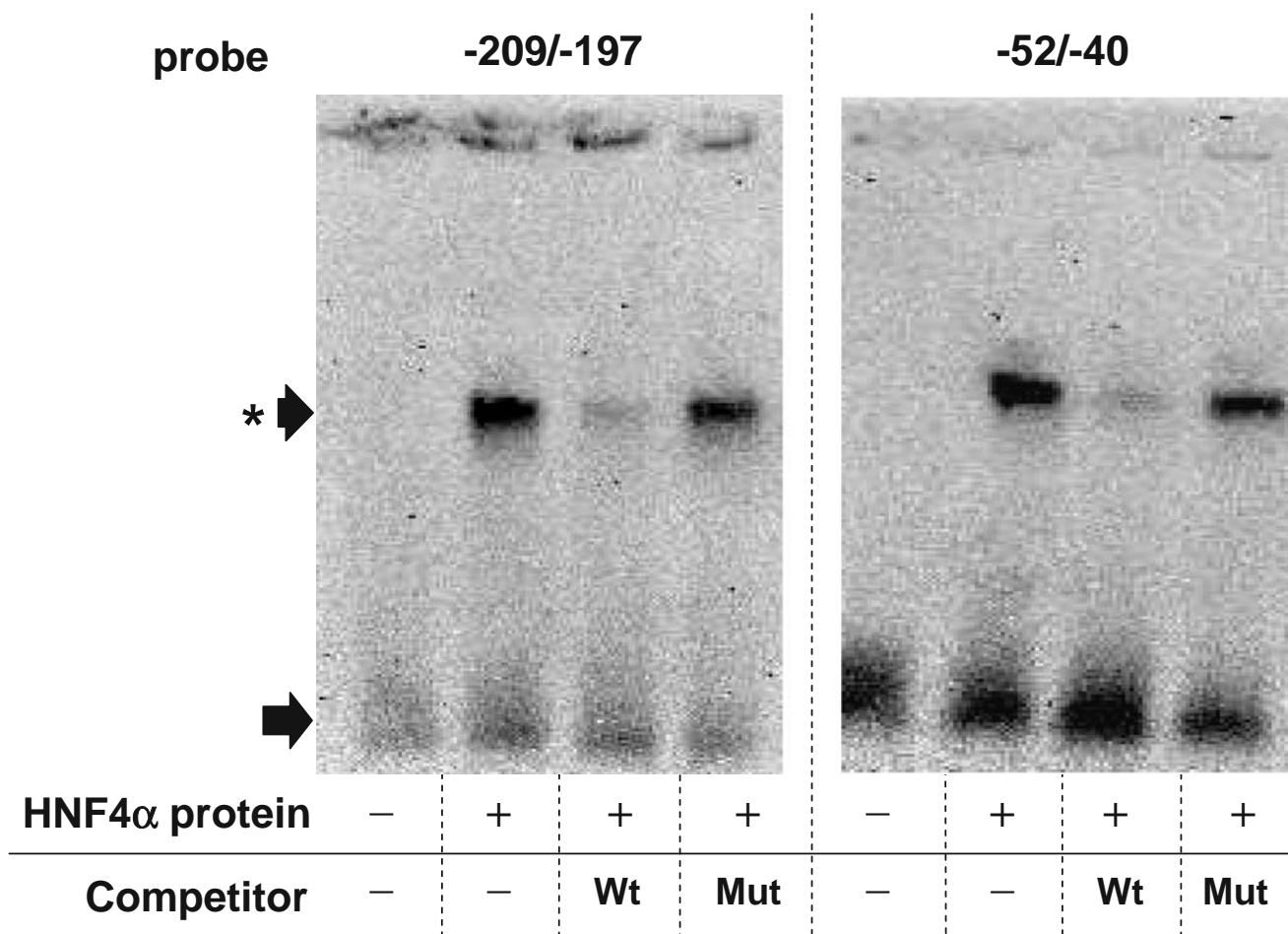


Fig. 4. Binding of HNF4 α on DR-1 sequences in human NPC1L1 promoters. EMSAs were performed for DR-1 elements in the NPC1L1 promoter. The arrow indicates the position of unreacted DNA probe and the asterisk with an arrowhead denotes the position of complex containing the DNA probe and HNF4 α . 5'-Flanking regions of human NPC1L1 gene (between -209 and -197 and between -52 and -40) were labeled with digoxigenin-11-ddUTP. The labeled DNA fragments were incubated with HNF4 α protein with or without 200-fold excess amount of unlabeled wild type or mutated DR-1 elements. The mixture was transferred to a gel and electrophoretically separated, then transferred onto a membrane by electroblotting. Digoxigenin-labeled complexes on the membrane were detected by anti-digoxigenin antibody.

crucial for the transcriptional regulation of the NPC1L1 gene. In fact, HNF4 α further transactivated the promoter activity of NPC1L1 along with SREBP2 (Fig. 1). Together with the detection of the direct binding of HNF4 α to the NPC1L1 promoter, NPC1L1 was found to be transcriptionally regulated by two HNF4 α binding elements in its promoter (Figs. 3 and 4). Moreover, HNF4 α specific-knock down significantly reduced the mRNA level of NPC1L1 and abolished the cholesterol-dependent regulation of NPC1L1 (Fig. 5). These results indicate that HNF4 α upregulates NPC1L1 transcription along with SREBP2 and this mechanism is important for the cholesterol-dependent transcriptional regulation of the NPC1L1 gene. Throughout the present study, we mainly used HepG2 cells, although Caco-2 cells have been the major cell-line to investigate the function of NPC1L1. In fact, we also performed the luciferase assay using Caco-2 cells and observed almost the same inducible patterns as HepG2 cells (data not shown). However, we found that both the endogenous expression level of NPC1L1 and the relative activities of luciferase assay were quite lower in Caco-2 cells than in HepG2 cells (data not shown). According to the fact that Caco-2 cells are derived from human colon, but not from

small intestine, and HepG2 cells are from human liver, it may be reasonable that the endogenous NPC1L1 expression level is much higher in HepG2 cells than Caco-2 cells. Indeed, human NPC1L1 is highly expressed in small intestine and liver, but not in colon. Based on these results, we decided to use HepG2 cells in our study.

Concerning the transcriptional regulation, HNF4 α is generally considered to bind to the direct repeat of AGGTCA-like hexamers separated by one or two nucleotides, referred to as DR-1 or DR-2. For instance, HNF4 α binds to the intergenic promoter region of ATP Binding Cassette transmembrane transporter G5/G8 (18), to the promoter of fatty acid binding protein via DR-1 (19), and to the promoter region of organic cation transporter-1 via DR-2 (12). However, since the HNF4 α binding site has a wide range of flanking, prediction of the functional HNF4 α site only by sequence analysis is practically impossible. In fact, although Davies *et al.* (2000) described the presence of putative HNF4 α binding sites in the NPC1L1 promoter (between -848 and -841, between -670 and -652, and between -617 and -601) based on the similarity of these sequences to that of DR-1 (10), all of them were located

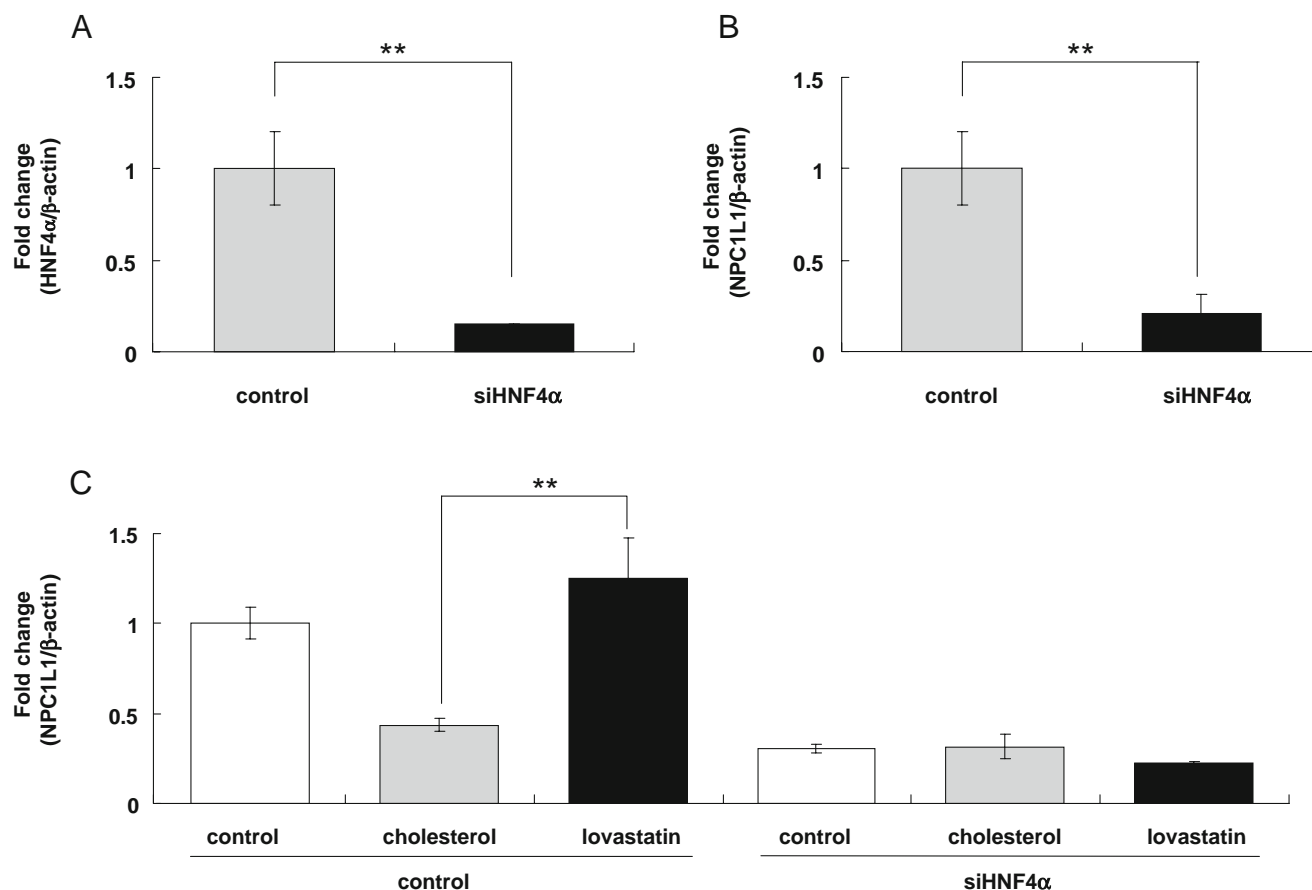


Fig. 5. Effect of HNF4 α specific knock-down in HepG2 cells. The effect of siRNA on the mRNA levels of endogenous HNF4 α (A) and NPC1L1 (B) was determined. HepG2 cells were transfected with the siRNAs targeting HNF4 α or an equal amount of control siRNAs using the RNAiMAX system. After 72 h, RNA was extracted and real-time PCR was performed. The fold changes were calculated as relative expression levels compared with that of control cells. Values are expressed as the mean \pm S.E. ($n=3$). Double asterisk significantly different from the control cells by Student's t test ($p < 0.01$). In addition, the effect of HNF4 α on the cholesterol-dependent expression of NPC1L1 was determined (C). HepG2 cells were transfected with the siRNAs targeting HNF4 α or an equal amount of control siRNAs using the RNAiMAX system. After 24 h, cells were exposed to 25-HCH or lovastatin, and then incubated for an additional 48 h. Values are expressed as the mean \pm S.E. ($n=3$). Double asterisk significantly different from the lovastatin-treated cells by Student's t test ($p < 0.01$).

upstream of -264 nt, and their deletion did not affect the transcription of NPC1L1 gene (Fig. 2). In addition to these regions, we have detected several putative DR-1 or DR-2 sites in the NPC1L1 promoter between -264 and transcriptional initiation site. The results of reporter gene assay and EMSA showed that only two of these sites (between -209 and -197 and between -52 and -40) act as functional HNF4 α binding elements (Figs. 3 and 4). Interestingly, one of the HNF4 α binding elements (between -52 and -40) is very close to the reported SRE (between -35 and -26) (9), which is consistent with the previous suggestion that cooperative transcriptional factors including HNF4 α bind to close cis-elements in the promoter region. For instance, HNF4 α and nuclear factor-Y (NF-Y) binding sites are located very close to each other on the promoter of human macrophage stimulating protein (20).

It was previously shown that NPC1L1 is activated by SREBP2 (9), although SREBP has three major isoforms, namely, SREBP1a, 1c and 2. Among them, SREBP1a and SREBP1c generally dominate the nutritional regulation of lipogenic genes, whereas SREBP2 is the key regulator of

cholesterol metabolism (21). In addition, although all three isoforms are known to be able to interact with HNF4 α , for the transcriptional regulation of the genes involved in cholesterol homeostasis, such as LDLR and HMG-CoA synthase, HNF4 α has been shown to solely interact with SREBP2 and suggested to accelerate the transcription of target genes by stimulating the transcriptional activity of SREBP2 (15,22). Indeed, for the genes encoding LDLR and HMG-CoA synthase, it is reported that HNF4 α does not transactivate the target genes by itself, but further enhances the inducible effect of SREBP2 (15). Results of the present study that the transcription of NPC1L1 is stimulated by HNF4 α only together with SREBP2, but not by HNF4 α alone (Figs. 1, 2 and 3) are also consistent with the previous belief (15). The mechanism for the effect of HNF4 α on stimulating the function of SREBP2 still remains to be clarified, although it was indicated that HNF4 α did not affect the affinity of SREBP2 to the promoters or the interaction between SREBP2 and the co-regulatory transcriptional factors, such as Sp1 and NF-Y (15). One of the possible explanations to account for the result of the stimulated NPC1L1 expression

by HNF4 α only together with SREBP2 may be that the endogenous expression levels of HNF4 α should be high enough in HepG2 cells under the present experimental conditions. Under such circumstances, the amount of active SREBP2 may be the rate-controlling factor for the NPC1L1 transcription, and consequently, increase in HNF4 α alone did not stimulate the transcription of NPC1L1. The result that the reduction in HNF4 α expression leads to the marked decrease in the endogenous expression level of NPC1L1 (Fig. 5) is consistent with this hypothesis.

Furthermore, we found the direct binding of HNF4 α to the sequences in the NPC1L1 promoter region (Fig. 4). Together with the results shown in Fig. 3 that the presence of the HNF4 α elements is necessary for the stimulation of SREBP2 activity by HNF4 α , it is suggested that HNF4 α may be cis-acting on the expression of NPC1L1, although the results of the previous report indicating the direct interaction between SREBP2 and HNF4 α imply the possibility of a trans-acting effect through SREBP2 (15). At the present moment, it is difficult to determine whether HNF4 α is a cis-acting element or trans-acting through SREBP2 on the NPC1L1 promoter.

Finally, the clinical implications of the regulation of NPC1L1 by HNF4 α should be discussed. It is well known that hyperlipidemia and diabetes are closely related syndromes. In addition, it has already been demonstrated that cholesterol absorption and NPC1L1 expression are increased under diabetic conditions (23,24). The present study demonstrating that NPC1L1 expression is regulated by HNF4 α , whose mutation is one of the well-established genetic risk factors for diabetes, may provide insight into mechanisms of the elevated cholesterol levels in diabetes. In fact, in the diabetic liver, the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1 α (PGC-1 α), which also acts as a coactivator of HNF4 α , is reported to be elevated, and that leads to the enhancement of HNF4 α activity (25). This alteration has been considered as the mechanism to explain the elevated production of glucose in the diabetic liver (26). Together with the results of the present study showing that the expression of NPC1L1 is positively regulated by HNF4 α , it is also possible that the increased NPC1L1 expression under diabetic conditions (23,24) may also be ascribed at least partly to the PGC-1 α mediated activation of HNF4 α . This regulation of NPC1L1 expression may be related to the elevated serum cholesterol levels in diabetes (27,28).

In conclusion, in the present study, we found that HNF4 α is a crucial modulator of NPC1L1, and it acts synergistically with SREBP2 on the NPC1L1 promoter. Furthermore, HNF4 α is crucial for cholesterol-dependent expression of NPC1L1. The fact that HNF4 α is responsible for increasing the circulating lipid levels (16) may be accounted for by the present finding that this transcription factor is involved in cholesterol homeostasis via NPC1L1. In addition, it is possible that HNF4 α is responsible for the increase in cholesterol absorption mediated by NPC1L1 in diabetes. The results of the present study may provide insight into the relationship between cholesterol metabolism and diabetes.

ACKNOWLEDGEMENTS

This work was supported by grants from The Japanese Ministry of Education, Science, Sports and Culture.

REFERENCES

1. M. van Heek, D. S. Compton, and H. R. Davis. The cholesterol absorption inhibitor, ezetimibe, decreases diet-induced hypercholesterolemia in monkeys. *Eur. J. Pharmacol.* **415**:79–84 (2001).
2. J. Patel, V. Sheehan, and C. Gurk-Turner. Ezetimibe (Zetia): a new type of lipid-lowering agent. *Proc. (Bayl. Univ. Med. Cent.)* **16**:354–358 (2003).
3. M. Garcia-Calvo, J. Lisnock, H. G. Bull, B. E. Hawes, D. A. Burnett, M. P. Braun, J. H. Crona, H. R. Davis, D. C. Dean, P. A. Detmers, M. P. Graziano, M. Hughes, D. E. Macintyre, A. Ogawa, K. A. O'Neill, S. P. Iyer, D. E. Shevell, M. M. Smith, Y. S. Tang, A. M. Makarewicz, F. Ujjainwalla, S. W. Altmann, K. T. Chapman, and N. A. Thornberry. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc. Natl. Acad. Sci. U. S. A.* **102**:8132–8137 (2005).
4. Y. Yamanashi, T. Takada, and H. Suzuki. Niemann-Pick C1-like 1 overexpression facilitates ezetimibe-sensitive cholesterol and beta-sitosterol uptake in CaCo-2 cells. *J. Pharmacol. Exp. Ther.* **320**:559–564 (2007).
5. S. W. Altmann, H. R. Davis, L. J. Zhu, X. Yao, L. M. Hoos, G. Tetzloff, S. P. Iyer, M. Maguire, A. Golovko, M. Zeng, L. Wang, N. Murgolo, and M. P. Graziano. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* **303**:1201–1204 (2004).
6. R. E. Temel, W. Tang, Y. Ma, L. L. Rudel, M. C. Willingham, Y. A. Ioannou, J. P. Davies, L. M. Nilsson, and L. Yu. Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *J. Clin. Invest.* **117**:1968–1978 (2007).
7. C. Duval, V. Touche, A. Tailleux, J. C. Fruchart, C. Fievet, V. Clavey, B. Staels, and S. Lestavel. Niemann-Pick C1 like 1 gene expression is down-regulated by LXR activators in the intestine. *Biochem. Biophys. Res. Commun.* **340**:1259–1263 (2006).
8. J. N. van der Veen, J. K. Kruit, R. Havinga, J. F. Baller, G. Chimini, S. Lestavel, B. Staels, P. H. Groot, A. K. Groen, and F. Kuipers. Reduced cholesterol absorption upon PPARdelta activation coincides with decreased intestinal expression of NPC1L1. *J. Lipid Res.* **46**:526–534 (2005).
9. W. A. Alrefai, F. Annaba, Z. Sarwar, A. Dwivedi, S. Saksena, A. Singla, P. K. Dudeja, and R. K. Gill. Modulation of human Niemann-Pick C1-like 1 gene expression by sterol: role of sterol regulatory element binding protein 2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**:G369–G376 (2007).
10. J. P. Davies, B. Levy, and Y. A. Ioannou. Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. *Genomics* **65**:137–145 (2000).
11. S. Jiang, T. Tanaka, H. Iwanari, H. Hotta, H. Yamashita, J. Kumakura, Y. Watanabe, Y. Uchiyama, H. Aburatani, T. Hamakubo, T. Kodama, and M. Naito. Expression and localization of P1 promoter-driven hepatocyte nuclear factor-4alpha (HNF4alpha) isoforms in human and rats. *Nucl. Recept.* **1**:5(2003).
12. M. Saborowski, G. A. Kullak-Ublick, and J. J. Elooranta. The human organic cation transporter-1 gene is transactivated by hepatocyte nuclear factor-4alpha. *J. Pharmacol. Exp. Ther.* **317**:778–785 (2006).
13. Y. Kamiyama, T. Matsubara, K. Yoshinari, K. Nagata, H. Kamimura, and Y. Yamazoe. Role of human hepatocyte nuclear factor 4alpha in the expression of drug-metabolizing enzymes and transporters in human hepatocytes assessed by use of small interfering RNA. *Drug Metab. Pharmacokinet.* **22**:287–298 (2007).
14. K. Ogasawara, T. Terada, J. Asaka, T. Katsura, and K. Inui. Hepatocyte nuclear factor-4{alpha} regulates the human organic anion transporter 1 gene in the kidney. *Am. J. Physiol. Renal Physiol.* **292**:F1819–F1826 (2007).
15. K. Misawa, T. Horiba, N. Arimura, Y. Hirano, J. Inoue, N. Emoto, H. Shimano, M. Shimizu, and R. Sato. Sterol regulatory element-binding protein-2 interacts with hepatocyte nuclear factor-4 to enhance sterol isomerase gene expression in hepatocytes. *J. Biol. Chem.* **278**:36176–36182 (2003).
16. G. P. Hayhurst, Y. H. Lee, G. Lambert, J. M. Ward, and F. J. Gonzalez. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol. Cell. Biol.* **21**:1393–1403 (2001).
17. M. Okuwaki, T. Takada, Y. Iwayanagi, S. Koh, Y. Kariya, H. Fujii, and H. Suzuki. LXR alpha transactivates mouse organic

- solute transporter alpha and beta via IR-1 elements shared with FXR. *Pharm. Res.* **24**:390–398 (2007).
18. K. Sumi, T. Tanaka, A. Uchida, K. Magoori, Y. Urashima, R. Ohashi, H. Ohguchi, M. Okamura, H. Kudo, K. Daigo, T. Maejima, N. Kojima, I. Sakakibara, S. Jiang, G. Hasegawa, I. Kim, T. F. Osborne, M. Naito, F. J. Gonzalez, T. Hamakubo, T. Kodama, and J. Sakai. Cooperative interaction between hepatocyte nuclear factor 4{alpha} and GATA transcription factors regulates ATP-binding cassette sterol transporters ABCG5 and ABCG8. *Mol. Cell Biol.* **27**:4248–4260 (2007).
 19. D. B. Jump, D. Botolin, Y. Wang, J. Xu, B. Christian, and O. Demeure. Fatty acid regulation of hepatic gene transcription. *J. Nutr.* **135**:2503–2506 (2005).
 20. A. Ueda, F. Takeshita, S. Yamashiro, and T. Yoshimura. Positive regulation of the human macrophage stimulating protein gene transcription. Identification of a new hepatocyte nuclear factor-4 (HNF-4) binding element and evidence that indicates direct association between NF-Y and HNF-4. *J. Biol. Chem.* **273**:19339–19347 (1998).
 21. D. Eberle, B. Hegarty, P. Bossard, P. Ferre, and F. Foufelle. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie.* **86**:839–848 (2004).
 22. T. Yamamoto, H. Shimano, Y. Nakagawa, T. Ide, N. Yahagi, T. Matsuzaka, M. Nakakuki, A. Takahashi, H. Suzuki, H. Sone, H. Toyoshima, R. Sato, and N. Yamada. SREBP-1 interacts with hepatocyte nuclear factor-4 alpha and interferes with PGC-1 recruitment to suppress hepatic gluconeogenic genes. *J. Biol. Chem.* **279**:12027–12035 (2004).
 23. S. Lally, D. Owens, and G. H. Tomkin. Genes that affect cholesterol synthesis, cholesterol absorption, and chylomicron assembly: the relationship between the liver and intestine in control and streptozotocin diabetic rats. *Metabolism* **56**:430–438 (2007).
 24. S. Lally, C. Y. Tan, D. Owens, and G. H. Tomkin. Messenger RNA levels of genes involved in dysregulation of postprandial lipoproteins in type 2 diabetes: the role of Niemann-Pick C1-like 1, ATP-binding cassette, transporters G5 and G8, and of microsomal triglyceride transfer protein. *Diabetologia* **49**:1008–1016 (2006).
 25. B. N. Finck, and D. P. Kelly. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J. Clin. Invest.* **116**:615–622 (2006).
 26. J. Rhee, Y. Inoue, J. C. Yoon, P. Puigserver, M. Fan, F. J. Gonzalez, and B. M. Spiegelman. Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **100**:4012–4017 (2003).
 27. N. L. Young, D. R. Lopez, and D. J. McNamara. Contributions of absorbed dietary cholesterol and cholesterol synthesized in small intestine to hypercholesterolemia in diabetic rats. *Diabetes* **37**:1151–1156 (1988).
 28. H. Gylling, J. A. Tuominen, V. A. Koivisto, and T. A. Miettinen. Cholesterol metabolism in type 1 diabetes. *Diabetes* **53**:2217–2222 (2004).