HNF4α Increases Liver-Specific Human ATP-Binding Cassette Transporter A1 Expression and Cholesterol Efflux to Apolipoprotein A-I in Response to Cholesterol Depletion

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Objective—Hepatic ATP-binding cassette transporter A1 (ABCA1) plays the major role in maintaining plasma high-density lipoprotein levels by producing cholesterol-accepting nascent high-density lipoprotein, whereas peripheral ABCA1 is responsible for releasing cellular cholesterol. We previously reported that in rodents, cholesterol depletion reduces ABCA1 expression in peripheral but not hepatic cells by increasing a liver-specific ABCA1 transcript via the sterol regulatory element-binding protein-2 system. However, the regulatory element is not conserved in humans. Here we investigated the mechanism of sterol-regulated human hepatic ABCA1 gene expression.

Methods and Results—ABCA1 mRNA variant type L3 is a novel and human-liver-specific transcript accounting for ∼25% of total ABCA1 mRNA in the liver and is induced by cellular cholesterol depletion. Specific knockdown or forced expression revealed that type L3 produces functional ABCA1 protein in cholesterol efflux. We identified a regulatory enhancer element for L3 expression lying within intron 3 of the human ABCA1 gene, to which HNF4α binds in response to cholesterol depletion. HNF4α knockdown abolished induction of liver-specific L3 and L2b transcripts (and consequently the liver-type response of ABCA1 expression to cellular cholesterol status) and diminished cholesterol efflux activity.

Conclusion—These findings indicate that HNF4α regulates human hepatic ABCA1 expression in response to cholesterol depletion. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: ABC transporter ■ gene expression ■ lipoproteins ■ cholesterol regulation

The ATP-binding cassette transporter A1 (ABCA1) is a plasma membrane transporter involved in cholesterol and phospholipid transport that plays a critical role in apolipoprotein A-I (apoA-I)–dependent biogenesis of high-density lipoprotein (HDL).1,2 ABCA1 gene mutations cause Tangier disease and familial HDL deficiencies characterized by a near absence of circulating HDL. The essential role of ABCA1 in HDL biogenesis was further demonstrated by studies that included ABCA1 overexpression and deletion in mice.3,4

Although ABCA1 is widely expressed in many tissues, gene-targeting studies in mice revealed that hepatic ABCA1 is responsible for approximately 80% of plasma HDL production.5 Further studies suggested that the role of hepatic ABCA1 in maintaining plasma HDL levels is distinct from that of extrahepatic ABCA1.6 Hepatic ABCA1 is critical for phospholipidating lipid-free apoA-I to generate cholesterol-poor early nascent HDL particles that in turn provide efficient cholesterol acceptors for peripheral cells, whereas ABCA1 in peripheral cells, such as macrophages and fibroblasts, mediates release of excess cellular cholesterol by generating mature HDL particles,7 probably in a sequential collaboration with ABCG1.7 The proposed roles of hepatic and peripheral ABCA1 are consistent with a classic model of the reverse cholesterol transport pathway, in which HDL transports excess cholesterol from peripheral cells to the liver, where cholesterol can be converted into bile acids for excretion.8 Hepatic and peripheral ABCA1 are under distinct gene regulation. ABCA1 expression in peripheral cells is upregulated by a liver X receptor (LXR)–driven promoter system in response to cholesterol loading.9 Conversely, cholesterol depletion or treatment of cells with statins that inhibit endogenous LXR ligand synthesis represses ABCA1 expression,9,10,11 collectively indicating that cellular cholesterol homeostasis is stringently controlled by the LXR-ABCA1 pathway in peripheral cells. In contrast, hepatic ABCA1 expression appears to be unaffected by sterols as suggested by the failure of cholesterol feeding of mice to elevate hepatic ABCA1 expression12,13 and the inability of cholesterol depletion to repress expression.14–17 In the preceding study using rodents,14 we identified a liver-specific ABCA1 mRNA variant and a corresponding promoter element driven by sterol regulatory element-binding protein-2 (SREBP-2), a

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transcription factor activated by cellular cholesterol depletion, in addition to the peripheral-type ABCA1 mRNA in the liver. Thus, cholesterol negatively and positively affects the liver-type SREBP-2 and peripheral-type LXR promoters, respectively, to control hepatic ABCA1 gene expression. This SREBP-2- and LXR-driven dual promoter system appears to ensure that hepatic ABCA1 expression is constant even under fluctuating cholesterol conditions and may be associated with ABCA1’s specific role in producing cholesterol acceptors in the reverse cholesterol transport pathway. Recently, microRNA-33 was shown to contribute to sterol-responsive ABCA1 gene regulation. MicroRNA-33, located within the gene encoding SREBPs, inhibits the expression of ABCA1 and makes a significant contribution to hepatic ABCA1 and plasma HDL levels in mice.18,19 Thus, multiple systems are likely to regulate hepatic ABCA1 expression.

In human hepatoma HepG2 cells, ABCA1 mRNA levels are slightly augmented on statin treatment,16,17 which suggests that a liver-type regulation also occurs in humans. However, the SREBP-2 binding element (sterol regulatory element) in the liver-type promoter is not conserved in humans because of no homologous sequence in rodent liver. Type L4a and L4b were similar to the human liver-type transcripts (Figure IA–IC in the online-only Data Supplement). Type L2a was less abundant compared with type L2a/b. Several minor transcripts, including those identified by Singaraja et al20 and Cavélier et al,21 are shown in Figure ID in the online-only Data Supplement. All products identified here were spliced according to the GT-AG splicing rule. Quantitative real-time RT-PCR (qRT-PCR) showed that newly identified type L3, L2b, or type P relative to that of total ABCA1 mRNA, we used variant-specific and universal RT-PCR probe/primer sets. The type L3 mRNA represented 26.2 ± 4.9% of total ABCA1 mRNA in human liver RNA preparations (n = 4), whereas type P and type L2b mRNA were estimated to be 33.4 ± 5.5% and 12.3 ± 4.8%, respectively, of total ABCA1 mRNA, indicating that type L3 is a novel and major ABCA1 transcript expressed in human liver. Type L3 was expressed in purified human hepatocytes at a higher level than that in the original total liver-cell preparations (Figure 2B).

Figure 1. Identification of alternative ATP-binding cassette transporter A1 (ABCA1) transcripts in human liver. A, 5’-RACE was performed on total RNA extracted from 4 human livers and THP-1 cells treated with sterols (10 μg/mL cholesterol plus 1 μg/mL 25-hydroxycholesterol; steroids = [S+] or compactin (50 μmol/L with 40 μmol/L mevalonate; steroids = [S–]) in the medium containing 10% delipidated serum for 16 hours. The amplified products were then subjected to electrophoresis in a 2% agarose gel. B, Structure of human ABCA1 gene and major transcripts. Black boxes indicate exons (ex) for type P transcript. Gray boxes indicate liver-variant specific exons.

**Methods**

An expanded Material and Methods section is available in the online-only Data Supplement. 5’-RACE polymerase chain reaction (PCR), plasmid construction, cell culture, RNA extraction and quantitative real-time reverse transcription (RT)-PCR, Northern and Western blot analyses, RNA interference, reporter gene assays, cholesterol efflux assays, chromatin immunoprecipitation assay, electrophoretic mobility shift assay, avidin biotin–conjugated DNA assay, lentivirus infection, and statistical analysis are described in detail in the online-only Data Supplement.

**Results**

Expression of Liver-Specific ABCA1 mRNA

**Variants in Human Liver Tissue and Hepatic Cells**

To identify liver-specific ABCA1 transcripts, we performed 5’-RACE on total RNA extracted from human liver and identified 4 major bands (Figure 1A) containing 5 major ABCA1 mRNA 5’-ends, which were designated types L2a, L2b, L3, and L4a/b (Figure 1B, bottom; details are shown in Figure 1A in the online-only Data Supplement), in addition to the previously described peripheral-type (type P) ABCA1 transcript.14 In contrast, peripheral THP-1 cells had only type P transcript. Type L3 is a novel transcript that lacks exon 1/exon 2 and has a newly identified 303-bp sequence (exon L3) upstream of exon 3. Exon L3 appears to be unique to humans because of no homologous sequence in rodent ABCA1 genes. Type L2a and L2b were homologous to rodent types L and L’, which we previously identified14 (Figure 1A–IC in the online-only Data Supplement). Type L4a and L4b were similar to the human liver-type transcripts previously identified20 and were expressed mainly in the liver (Figure II in the online-only Data Supplement). Type L2b was less abundant compared with type L2a. Several minor transcripts, including those identified by Singaraja et al20 and HNF4a regulates expression of liver-specific ABCA1 transcripts in response to cholesterol depletion.

**Table 1.** Identification of alternative ATP-binding cassette transporter A1 (ABCA1) transcripts in human liver. A, 5’-RACE was performed on total RNA extracted from 4 human livers and THP-1 cells treated with sterols (10 μg/mL cholesterol plus 1 μg/mL 25-hydroxycholesterol; steroids = [S+] or compactin (50 μmol/L with 40 μmol/L mevalonate; steroids = [S–]) in the medium containing 10% delipidated serum for 16 hours. The amplified products were then subjected to electrophoresis in a 2% agarose gel. B, Structure of human ABCA1 gene and major transcripts. Black boxes indicate exons (ex) for type P transcript. Gray boxes indicate liver-variant specific exons.

Cavelier et al,21 are shown in Figure ID in the online-only Data Supplement. All products identified here were spliced according to the GT-AG splicing rule.
Type L3 Is a Major ABCA1 Transcript Producing a Functional ABCA1 Protein

We investigated whether type L3 mRNA encodes a functional ABCA1 protein using a short interfering RNA (siRNA) specific to the type L3 transcript. In cholesterol-depleted JHH-5 and Hep3B cells, transfection of type L3-specific siRNA effectively diminished type L3 mRNA expression without decreasing type L2b and type P mRNA levels (Figure 3A). Notably, specific knockdown of type L3 mRNA caused a significant reduction in the total ABCA1 mRNA (Figure 3A) and protein levels (Figure 3B). To exclude off-target effects, we repeated the experiments using 3 other siRNAs specific to type L3, and we confirmed that these siRNAs also effectively diminished type L3 mRNA and decreased total ABCA1 mRNA and protein expression (Figure 3D and Figure IV). Northern blot analysis confirmed the L3 mRNA knockdown and an accompanying decrease in total ABCA1 mRNA band (Figure 3C). Furthermore, siRNA treatment caused an ~45% decrease in apoA-I-dependent [3H]cholesterol efflux from JHH-5 cells under cholesterol-depleted conditions (Figure 3E). Also, 3 siRNAs to type L3 significantly decreased the mass of free cholesterol efflux under normal culture conditions (Figure 3F). Although type L3 ABCA1 mRNA lacks exon 2, which includes a known translation start codon, ATG9,23 (Figure 1B and Figure III in the online-only Data Supplement), the L3 transcript could be induced at the predicted start codon located at the 3’-end of exon L3 (Figures IC and III in the online-only Data Supplement). We constructed an expression vector of type L3 ABCA1 fusing green fluorescent protein to the C terminus and obtained JHH-5 cells stably expressing L3-ABCA1–green fluorescent protein, in which marked increases of ABCA1 expression and cholesterol efflux activity were observed (Figure 3G and 3H). Together, these results clearly show that type L3 is a major transcript producing a functional ABCA1 protein in the liver.

Identification of the Type L3 Promoter and Its Cholesterol-Responsive Enhancer Elements

To explore the mechanisms by which type L3 transcripts were increased by cholesterol depletion, we searched for a possible promoter region for the L3 transcript in the region upstream of the putative transcription initiation site (+1) for type L3 mRNA (1010 or 4208 bp) (Figure 4A and 4B, Luc1–Luc3; Figure VI in the online-only Data Supplement). Although these regions did not have basal promoter activity and were unresponsive to cholesterol depletion in a luciferase assay, a reporter construct containing an element from +426 to +480 did show...
Figure 3. Specific knockdown or forced expression of type L3 mRNA modulates total ATP-binding cassette transporter A1 (ABCA1) mRNA and protein expression and apolipoprotein A-I (apoA-I)–dependent cholesterol efflux. A to F, JHH-5 or Hep3B cells were transfected with either short interfering RNA (siRNA) against ABCA1 type L3 (A, B, and E, #1; C to F, #1, 2, 3, or 4) or a negative control (ctrl, con) siRNA. After 32 hours, cells were treated with compactin (50 and 40 μmol/L mevalonate: sterols−) for 16 hours. A, Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) analysis of total and variant ABCA1 mRNA levels. Data were normalized with 18S rRNA and represent means±SD (n=3). B, Northern blot analysis of total and variant ABCA1 mRNA levels using ABCA1 total and L3-specific probes. Bar graphs represent means±SD (n=4). E, F, and H, Efflux of [3H]cholesterol (E) or cholesterol mass (F and H) from the cells to the media was determined as described in Materials and Methods. G and H, JHH-5 cells stably expressing ABCA1 type L3–green fluorescent protein (GFP). G, Immunoblot analysis for ABCA1, GFP, and the loading control anti-β-actin. Bar graphs represent means±SD (n=3). H, Efflux of cholesterol mass from the cells to the media was determined. Data represent means±SD (n=3). *P<0.05 vs control siRNA-transfected (A–F) or mock-transfected (H) cells.
basal transcriptional activity and caused mild activation on cholesterol depletion (Figure 4B, Luc5), albeit at a level that is likely insufficient to produce a full response to sterols. The promoter activities of several genes are often regulated by distal enhancers. Sequence alignment of 5 mammalian ABCA1 genes identified a highly conserved region in intron 3 (positioned between +3103 and +3378 in humans) (Figure 4A; Figures VI and VII in the online-only Data Supplement). Therefore, we examined whether this region functions as an enhancer for the ABCA1 type L3 promoter by inserting the conserved region of intron 3 into the 3'-end of the poly A site (Figure 4A). We found that insertion of this region effectively enhanced type L3 promoter activity, and this activity was further amplified by cholesterol depletion (Figure 4C, Luc7).
Furthermore, introducing this region into a reporter plasmid driven by the SV40 promoter instead of the type L3 promoter potentiated both basal promoter activity and a sterol response (Figure 4D, Luc9). These results suggest that the conserved region in ABCA1 intron 3 functions as a distal enhancer of the type L3 promoter that could be responsible for upregulation under sterol-depleted conditions.

To identify the sterol-responsive elements in this enhancer region, we performed a deletion analysis in this region. Deleting the 5’-end up to +3171 enhanced the promoter activity and sterol-mediated response (Luc10), whereas further deletion greatly diminished these effects (Figure 4E, Luc11–Luc14). The region between +3172 and +3183 contains a palindromic sequence box (P-Box) (Figure 4E, upper scheme). In a similar fashion, 3’-end deletions up to +3260 enhanced the promoter activity and the response to sterol depletion (Luc16), whereas deletion up to +3247 (Luc17) markedly decreased the response, and further deletion up to +3230 (Luc18) completely abolished these effects. Accordingly, a reporter containing the region from +3172 to +3259 showed a strong response to sterol depletion (Figure 4E, Luc19). Within this responsive region, deletion of either the 3’-end up to +3247 or the region between +3229 and +3241 significantly repressed the sterol-mediated response (Figure 4E, Luc20, Luc21). These results suggest that the P-box and the 2 elements (+3247 to +3259, +3229 to +3241) are required for the sterol-mediated response in the distal enhancer region in ABCA1 intron 3. A homology search detected consensus binding sequences for various transcription factors, suggesting that these elements (+3230 to +3243 and +3224 to +3256) could be binding sites for liver-enriched transcription factors such as HNF1 and HNF4, respectively (http://tfbind.ims.u-tokyo.ac.jp/; Figure 4E, upper scheme).

HNF4α Regulates Type L3 Promoter Activity Through Binding to the Distal Enhancer Elements

The role of liver-specific transcription factors HNF1α and HNF4α in the sterol-responsive distal enhancer activity within ABCA1 intron 3 was investigated. Whereas HNF4α expression effectively augmented type L3 promoter-enhancer activity in a distal enhancer region-dependent manner, HNF1α produced only minimal enhancement (Figure 5A). Expression of a dominant negative form of HNF4α lacking the AF-2 domain repressed its promoter-enhancing activity, especially under cholesterol-depleted conditions (Figure 5B).

HNF4α expression also augmented the activity of a SV40 promoter-driven reporter gene containing the ABCA1 enhancer region (Figure 5C, Luc22) but did not activate a construct that lacked the putative HNF4-binding site (Luc23). Deletion of the P-box (Luc24) or putative HNF1 site (Luc25) did not affect this HNF4α-mediated augmentation. In addition, the activity of a reporter gene containing 4 tandem repeats of the HNF4-response element was enhanced by HNF4α expression (Luc26). These results indicate that the HNF4-element, but not the P-box nor putative HNF1-binding site, is critical for HNF4α-mediated activation of ABCA1 expression.

We performed a chromatin immunoprecipitation assay to investigate whether endogenous HNF4α protein associates with chromatin via this putative HNF4-binding site. Clear bands were detected when chromatin from cholesterol-depleted JHH-5 cells was immunoprecipitated with antibodies against HNF4α, indicating that a tight association of endogenous HNF4α protein with the conserved intron 3 region was induced by cholesterol depletion (Figure 5E). Additionally, an in vitro DNA binding assay confirmed the association of HNF4α with this site. Endogenous HNF4α in lysates of cholesterol-depleted cells reacted with biotin-conjugated oligodeoxynucleotides corresponding to the ABCA1 region from +3223 to +3262 that includes the HNF4-response element (#1), but not oligonucleotides that lacked this element (#2) (Figure 5F). Furthermore, electrophoretic mobility shift assay also confirmed the association of HNF4α with this binding site (Figure VIII in the online-only Data Supplement). These results indicate that the putative HNF4-binding site on ABCA1 intron 3 is authentic.

HNF4α is Required for Expression of Liver-Specific ABCA1 mRNA and Protein in Response to Sterol Depletion

The role of HNF4α in liver-type ABCA1 mRNA variant expression was investigated using siRNA knockdown. In JHH-5 cells, 3 siRNAs against HNF4α effectively reduced HNF4α mRNA and protein expression (Figure 6A and 6B). HNF4α knockdown resulted in the reduced expression of type L3 and also type L2b but not type P transcripts under sterol-depleted conditions (Figure 6A, black bars). Consistently, expression of total ABCA1 mRNA and protein was reduced by HNF4α knockdown under sterol-depleted conditions, (Figure 6A and 6B). Reduction of type L3 and total ABCA1 mRNA was also confirmed by Northern blot (Figure V in the online-only Data Supplement). In the presence of cholesterol, however, HNF4α knockdown did not affect the expression of ABCA1 transcripts (Figure 6A, white bars), although 1 siRNA (#1) induced type P and total ABCA1 mRNA expression probably because of an off-target effect. Similar results were obtained with another human hepatic cell line Hep3B (Figure IXA and IXB in the online-only Data Supplement). In agreement with these results, HNF4α knockdown in JHH-5 and Hep3B cells reduced apoA-I-mediated cholesterol efflux under cholesterol-depleted conditions (Figure 6C; Figure IXC in the online-only Data Supplement). Conversely, forced expression of HNF4α increased the level of type L3 and L2b transcripts, leading to a significant increase in total ABCA1 mRNA expression and apoA-I-mediated cholesterol efflux (Figure X in the online-only Data Supplement). We also confirmed that HNF4α knockdown markedly reduced the type L3 promoter-enhancer activity (Figure XIIA in the online-only Data Supplement) and the HNF4-binding site-dependent intron 3 enhancer activity (Figure XIIB in the online-only Data Supplement) in sterol depleted cells but did not efficiently modulate the expression of LXRα, LXRβ, and micro-RNA-33, which are regulatory genes of ABCA1 transcript (Figure XI in the online-only Data Supplement). These results indicate that HNF4α is required for liver-specific ABCA1 transcript (L3 and L2b) expression and liver-type response of ABCA1 mRNA and protein expression on cholesterol depletion.
Discussion

The liver is the major source of apoA-I, and hepatic ABCA1 is responsible for producing the majority of plasma HDL. Hepatic ABCA1 has a specific role in producing early nascent HDL particles, and its genetic regulation is distinct from extrahepatic ABCA1. In this study, we investigated the regulation of ABCA1 expression in human liver cells and discovered a liver-specific regulatory system in humans. Among the liver-specific variants (types L2a/b, L3, L4a/b), type L3 is a novel ABCA1 transcript containing a human-specific exon L3 and is a major transcript accounting for 25% of total ABCA1 mRNA in human liver.

The ABCA1 protein translated from type L3 transcript lacks the amino-terminal 21 amino acids, because the first ATG codon resides in the 3′-end of exon L3 (Figure III in the online-only Data Supplement). Although early studies showed that an ABCA1 expression construct lacking the 60 amino-terminal amino acids does not produce a functional protein, recent studies have suggested that the amino-terminal domain is crucial for maintaining HDL particle integrity and cholesterol efflux. HNF4α regulates ATP-binding cassette transporter A1 (ABCA1) type L3 promoter-enhancer activity by binding to the distal enhancer element. A to D, JHH-5 (A, B, and D) or HepG2 (C) cells were transfected with the indicated reporter plasmids and control plasmid phRL-SV40 in the absence or presence of HNF4α or HNF1α expression vectors. A, C, and D, After 48 hours, the firefly luciferase (Luc) activity in cell lysates was measured and normalized with Renilla luciferase activity. B, After 30 hours of transfection, the cells were treated under sterols+ or sterols− conditions as in Figure 2B for 16 hours. DN indicates dominant negative. Data represent means±SD (n=3). SV40 indicates SV40 promoter. E and F, JHH-5 cells were treated under sterols+ or sterols− conditions for 16 hours. E, Cells were subjected to chromatin immunoprecipitation (IP) assay using the indicated antibodies or control IgG. The immunoprecipitated region containing HNF4α-response elements within the intron 3 enhancer region was amplified with polymerase chain reaction (PCR) using specific primers as described in Materials and Methods. The amplified PCR products were subjected to electrophoresis in a 2% agarose gel. Input indicates that genomes extracted from 2% of total lysates were amplified with PCR. F, The cell lysates were subjected to avidin biotin–conjugated DNA assays using 2 biotin-conjugated nucleotides (#1 or #2) as indicated in the top panel and, together with an aliquot of the lysates (input), immunoblotted (IB) with anti-HNF4α antibody. The arrow and asterisk indicate HNF4α protein and nonspecific bands, respectively.

Figure 5. HNF4α regulates ATP-binding cassette transporter A1 (ABCA1) type L3 promoter-enhancer activity by binding to the distal enhancer element. A to D, JHH-5 (A, B, and D) or HepG2 (C) cells were transfected with the indicated reporter plasmids and control plasmid phRL-SV40 in the absence or presence of HNF4α or HNF1α expression vectors. A, C, and D, After 48 hours, the firefly luciferase (Luc) activity in cell lysates was measured and normalized with Renilla luciferase activity. B, After 30 hours of transfection, the cells were treated under sterols+ or sterols− conditions as in Figure 2B for 16 hours. DN indicates dominant negative. Data represent means±SD (n=3). SV40 indicates SV40 promoter. E and F, JHH-5 cells were treated under sterols+ or sterols− conditions for 16 hours. E, Cells were subjected to chromatin immunoprecipitation (IP) assay using the indicated antibodies or control IgG. The immunoprecipitated region containing HNF4α-response elements within the intron 3 enhancer region was amplified with polymerase chain reaction (PCR) using specific primers as described in Materials and Methods. The amplified PCR products were subjected to electrophoresis in a 2% agarose gel. Input indicates that genomes extracted from 2% of total lysates were amplified with PCR. F, The cell lysates were subjected to avidin biotin–conjugated DNA assays using 2 biotin-conjugated nucleotides (#1 or #2) as indicated in the top panel and, together with an aliquot of the lysates (input), immunoblotted (IB) with anti-HNF4α antibody. The arrow and asterisk indicate HNF4α protein and nonspecific bands, respectively.
protein in cells, specific siRNAs against type L3 efficiently reduced ABCA1 protein and cholesterol efflux. Moreover, transfection of L3-ABCA1 expression vector into JHH-5 cells augmented ABCA1 protein and cholesterol efflux activity, demonstrating that the L3-derived ABCA1 variant protein is stable and functionally active.

HNF4α Regulates Hepatic ABCA1 Gene Expression in Humans

When cellular cholesterol is depleted, the liver-specific type L3 and L2b transcripts are induced and positively regulate ABCA1 protein expression (Figure XIII in the online-only Data Supplement). We previously discovered that SREBP-2, a transcription factor activated on cellular cholesterol depletion, upregulates the rodent liver-type (type L) ABCA1 promoter. However, the SREBP-2 binding element (sterol regulatory element) in the rat promoter is not conserved in humans (Figure XIVA in the online-only Data Supplement), and the human type L2 promoter containing this region is not activated by cholesterol depletion (Figure XIVB in the online-only Data Supplement). In humans, we found a critical role of the liver-enriched transcription factor HNF4α in hepatic ABCA1 expression. Knockdown of HNF4α abolished inductions of L3 and L2b mRNA and, conversely, forced expression of HNF4α in JHH-5 cells increased them. Altered L3 and L2b mRNA levels led to substantial changes in total ABCA1 mRNA and protein expression and in cholesterol efflux activity. Thus, HNF4α is required for the induction of liver-specific ABCA1 transcripts, including types L3 and L2b, and thereby modulates ABCA1 expression and HDL generation.
We identified a regulatory enhancer element for L3 expression lying within intron 3 of the human ABCA1 gene, to which HNF4α binds and at which HNF4α augments the L3 promoter-enhancer activity in response to cholesterol depletion. Because HNF4α expression was increased on cholesterol depletion and HNF4α knockdown abolished the L3 and L2b induction, it is likely that the HNF4α level or activity (via dephosphorylation) determine the sterol-responsive L3 and L2b expression. However, it is more likely that along with HNF4α, several transcription factor(s) cooperatively regulate the L2 and L3 expression, because we identified several elements (and region) responding to cholesterol depletion in the intron 3-enhancer and the L3 promoter (Figure 4E). In addition, in human JHH-5 cells, SREBP-2 knockdown partly diminished the L3 and L2b induction on sterol depletion (Figure XV in the online-only Data Supplement). Thus, SREBP2 partially regulates human hepatic ABCA1 expression through distinct mechanism from that in rodents. Although decreased HNF4α expression (Figure XV in the online-only Data Supplement) may be responsible for this effect, it is possible that SREBP-2 directly (via some unknown sterol regulatory element) or indirectly (via interaction with HNF4α) regulates L3 and L2b mRNA expression. Decreased type P mRNA expression by SREBP-2 knockdown is probably due to the decreased supply of endogenous ligands for LXR.27

HNF4α knockdown resulted in the reduced expression of types L3 and L2b but not type P. Although we have confirmed that neither LXR regulating type P promoter nor microRNA-33 regulating ABCA1 mRNA stability was affected by HNF4α knockdown, further investigation on chromatin-loop or transcription factors is needed to understand the mechanism.

Because the HNF4 binding site located in ABCA1 intron 3 is conserved among mammals (Figure III in the online-only Data Supplement), HNF4α may also regulate the liver-type ABCA1 transcript(s) in rodents. However, we could not confirm this because of the unavailability of variant-specific primers for rodent type L mRNA that corresponds to human type L2a. In addition, a rodent variant corresponding to human type L3 may not be expressed in rodent liver because exon L3 is not conserved in rodents. Studies have shown that acute loss of HNF4α (for 6–7 days) in mouse liver reduced ABCA1 mRNA expression,28 although Cre-loxP knockdown did not affect the level.29 Thus, the detailed mechanisms that regulate liver-specific ABCA1 gene expression may differ between humans and rodents. However, hepatic ABCA1 gene expression is ensured by stimulating liver-specific systems on cholesterol depletion, a response that is conserved among humans, mice, and rats.

HNF4α is a highly conserved member of the nuclear receptor superfamily.26 Loss-of-function mutations in the human HNF4α gene cause the disorder maturity onset diabetes of the young (MODY1).20 HNF4α is required for early liver development and expression of many liver-specific genes, including those involved in lipoprotein and cholesterol/bile acid metabolism.26,30 For example, HNF4α regulates expression of MTP, apolipoprotein B, CII, and CIII, genes involved in very-low-density lipoprotein production; cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme of bile acid biosynthesis from cholesterol; and ABCG5/ABCG8, transporters involved in cholesterol/sterol excretion from the liver.32 Together with these genes, HNF4α-regulated ABCA1 gene expression may cooperatively control hepatic cholesterol elimination to maintain cholesterol homeostasis. A recent study has shown that HNF4α-siRNA knockdown in mouse liver reduces expression of many lipid metabolism genes, including ABCA1, leading to decreases in HDL and very-low-density lipoprotein in plasma.28

**Dual System Regulates Human Hepatic ABCA1 Gene Expression in Response to Sterols**

In the model of reverse cholesterol transport pathway, the liver plays a role in excreting cholesterol transported from peripheral tissues.3 The liver is also the major site of production of HDL, and hepatic ABCA1 is responsible for maintaining plasma HDL by producing a precursor form of HDL,5,6 which could accept cholesterol from peripheral cells. When cholesterol accumulates in the liver, the type P ABCA1 transcript is induced by the LXR promoter.14 However, this rise is counterbalanced by the reduced expression of liver-type L3 and L2a/b transcripts (Figure XIII in the online-only Data Supplement). The dual system may prevent overshooting ABCA1 expression and retransplant of cholesterol to peripheral tissues. On cholesterol depletion, type P ABCA1 expression is reduced, but enhanced type L3 and L2a/b expression may compensate for this reduction. Thus, the dual system ensures constant expression of ABCA1 and HDL production in the human liver even under fluctuating cholesterol conditions. Recent studies show that microRNA-33 regulates hepatic ABCA1 expression and plasma HDL levels in mice.18,19 ABCA1 expression also undergoes posttranscriptional regulation.23 Because hepatic ABCA1 plays the major role in maintaining plasma HDL levels, it seems reasonable that hepatic ABCA1 expression would be stringently regulated by multiple sterol-responsive systems.

**Regulation of Human Hepatic ABCA1 as a Novel Strategy to Prevent Atherosclerosis**

Statins are widely used to treat hypercholesterolemia and raise plasma HDL cholesterol independently of reducing low-density lipoprotein cholesterol.34 Several studies have shown that statins upregulate hepatic ABCA1 mRNA expression in human hepatoma cells and mouse liver,15–17 whereas statins repress peripheral ABCA1 mRNA expression by depleting endogenous LXR ligands.10,11 Our findings indicate that the liver-specific ABCA1 variants, including type L3 and L2a/b (or L in rodents), are responsible for statin-induced ABCA1 expression and may be associated with increased plasma HDL levels.

Our current study clarified for the first time a mechanism by which human hepatic ABCA1 expression is regulated. HDL cholesterol levels are inversely correlated with cardiovascular risk. Because hepatic ABCA1 has the largest impact on plasma HDL levels5,6 and is the most promising therapeutic target, our findings provide a basis for developing novel drugs to control plasma HDL levels and prevent atherosclerosis.
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References

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Supplement Material

Methods

Materials. Anti-HNF4α polyclonal antibody (H-171) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin monoclonal antibody and protease inhibitor cocktail were from Sigma Chemical Co. (St. Louis, MO). Anti-ABCA1 antibodies were from abcam. Human normal liver total RNA sample #1 (male Caucasian, age 51) was obtained from Clontech Laboratories (Mountain View, CA), and RNA sample #2 (male Asian, age 64), #3 (male Asian, age 24) and #4 (male Asian, age 26) were from Bio Chain Institute (Hayward, CA). Human total liver cells #5 (H0911A2: male Caucasian, age 32) and #6 (H1068A2: male Caucasian, age 47) were freshly isolated, and from which hepatocytes were purified with Percoll-gradient, and both were supplied by SciKon Innovation, Inc. (Chapel Hill, NC). The multiple human tissue cDNA (MTC™) panel I was from Clontech (Lot No. 7080213).

Cell culture. HepG2 cells (obtained from American Type Culture Collection (ATCC), Manassas, VA) were maintained in DMEM containing 10% fetal bovine serum (FBS) while Hep3B cells (obtained from ATCC) were maintained in DMEM containing 10% FBS and 1% non essential amino acids. JHH-5 cells (established by Dr. Nagamori² and obtained from Japanese Collection of Research Bioresources, Osaka, Japan) were cultured in William’s E medium containing 10% FBS. THP-1 cells were maintained RPMI medium containing 10% FBS. THP-1 cell differentiation was induced by phorbol 12-myristate 13-acetate (PMA) for 72 h. The differentiated cells were cultured in RPMI medium containing 0.2% bovine serum albumin (BSA) for 24 h before use in experiments³.

Expression and reporter plasmid construction. The expression plasmids pCMV-3Flag-HNF4α WT (aa 1-474) and pCMV-Flag-HNF4α DN lacking aa 359-474 of HNF4α were generated by PCR. The expression plasmids pcDNA3.1-ABCA1 type L3-GFP was constructed by inserting a fragment coding for the ABCA1 protein translated from the predicted start codon located at 3'-end of exon L3, fusing enhanced green fluorescence protein (EGFP) at the C-terminus, into pcDNA3.1. The lentiviral expression plasmids pCSII-EF-3Flag-HNF4α was constructed by inserting a fragment coding for HNF4α into pCSII-EF-MCS-IRE2-Venus (RIKEN, Saitama, Japan). The reporter plasmids pABCA1 type L3-Luc and pSV40-ABCA1 intron 3-Luc were generated by ligating the human ABCA1 type L3 promoter region (-4208 to +744) with pGL3-basic (Promega) or the human ABCA1 intron 3 (+3103 to +3378) with pGL3-promoter (Promega), respectively. To obtain p(HNF4)4-Luc, hybridized cDNA for four tandem repeats of the HNF4-response element from the human ABCA1 intron 3 were ligated with the pGL3-promoter. All constructs were verified by DNA sequencing.

5’-RACE PCR. We employed RNA ligase-mediated RACE (RLM-RACE) with 3’-primers specific for exon 4-5 of the human ABCA1 gene (Fig. 1B upper) to obtain the 5’-ends of full-length transcripts, and to exclude artificially truncated transcripts. In brief, we used the GeneRacer kit (Invitrogen, Carlsbad, CA), and total RNAs were treated with calf intestinal phosphatase (CIP) to remove 5’ phosphates from truncated mRNA and non-mRNA, which prevented their subsequent ligation with the GeneRacer™ RNA Oligo. The RNA mixture was then treated with tobacco acid pyrophosphatase (TAP) to remove the 5’ cap structures from intact, full-length mRNA. The GeneRacer RNA Oligos were ligated to the 5’ ends of the mRNA using T4 RNA ligase. To obtain 5’ ends, the first-strand cDNA was amplified using the GeneRacer 5’ primer and a reverse gene-specific primer, in this case specific for the human ABCA1 exon 5 (5’-GAGAACAGGGCGAGCCACAATGGAATTG-3’). The amplified products were subcloned and sequenced.

RNA extraction and quantitative real-time RT-PCR. Total RNA was extracted with an RNeasy Mini Kit using on-column deoxyribonuclease digestion to eliminate genomic DNA contamination according to the manufacturer’s instructions (QIAGEN, Valencia, CA). Quantitative real-time RT-PCR was performed with an ABI Prism 7300 sequence detection system using TaqMan one-step RT-PCR Master Mix Reagent Kit (Applied Biosystems, Foster City, CA) with the TaqMan probes/primers as follows: human total ABCA1, forward: 5’-TGGCCAGTCCAGTAATGGTCTGT-3’; reverse:
5’-AAGCGAGATAATGGTGCCGATT-3’;  
5’-FAM-ACACCTGGAGAGAAGCTTTCACCCAGTAACC-TAMRA-3’; human type P ABCA1, 
forward: 5’-GGCGCTTTGCTCCTTGTTT-3’; reverse: 5’-TCTTTTCTCCTACCCCTTGGACA-3’; 
probe: 5’-FAM-ACACCTGGAGAGAAGCTTTCACCCAGTAACC-TAMRA-3’; human type P ABCA1, 
forward: 5’-GGTGTATGGCTTTGCAGCAA-3’; reverse: 5’-CCGTGGCTGGTCATTAACTGA-3’; 
probe: 5’-FAM-CAATGAGTATGGCTGTTTCCCCTCCTGCTGTTTAACTCTTCCTGCTGCTGCT-3’; 
human type L2b ABCA1, forward: 5’-GGCGCTTTGCTCCTTGTTT-3’; reverse: 5’-TCTTTTCTCCTACCCCTTGGACA-3’; 
probe: 5’-FAM-CAATGAGTATGGCTGTTTCCCCTCCTGCTGCTGCTGCTGCTGCT-3’; 
Northern blot analysis. Poly A-tailed mRNA fractions were was separated in a 0.8% agarose gel 
containing 2% formaldehyde and transferred onto a nylon membrane Hybond-N+ (Amersham 
Biosciences). The membranes were hybridized with a 32P-labeled ABCA1 total and L3 cDNA probes 
and the specific mRNA on the filter was detected by autoradiography using a Bioimage analyzer, BAS 
2500 (Fuji, Tokyo, Japan). A universal ABCA1 total probe and an L3-specific probe were designed 
based on the sequences within exon 10-13 and exon L3, respectively.

RNA interference. All hepatic cells were transiently transfected with gene-specific Stealth™ Select 
RNAi or Stealth RNAi negative control high GC (Invitrogen, Carlsbad, CA) using lipofectamine 
RNAi MAX reagent (Invitrogen). The Stealth RNAi sequences used were: human type L3 ABCA1 
sense1 5’-AACCCAGGCACACUCUCUCUGUAGC-3’, antisense1 5’-AACCCAGGCACACUCUCUCUGUAGC-3’, 
sense2 5’-GUCCGCCGCAAGUGCGCGGCUUUU-3’, antisense2 5’-GUCCGCCGCAAGUGCGCGGCUUUU-3’, 
sense3 5’-UGGCGCGGCAAGUGCGCGGCUUUU-3’, antisense3 5’-UGGCGCGGCAAGUGCGCGGCUUUU-3’, 
sense4 5’-AAGGGGAGUGCGCUUUCUCCUGWCU-3’, antisense4 5’-AAGGGGAGUGCGCUUUCUCCUGWCU-3’, 
human sense1 5’-AACCCAGGCACACUCUCUCUGUAGC-3’, antisense1 5’-AACCCAGGCACACUCUCUCUGUAGC-3’, 
sense2 5’-GUCCGCCGCAAGUGCGCGGCUUUU-3’, antisense2 5’-GUCCGCCGCAAGUGCGCGGCUUUU-3’, 
sense3 5’-UGGCGCGGCAAGUGCGCGGCUUUU-3’, antisense3 5’-UGGCGCGGCAAGUGCGCGGCUUUU-3’, 
sense4 5’-AAGGGGAGUGCGCUUUCUCCUGWCU-3’, antisense4 5’-AAGGGGAGUGCGCUUUCUCCUGWCU-3’, 

For microRNA assays, total RNA was extracted from cells using miRNA miRNA isolation 
kit (Ambion, Invitrogen) and reverse transcribed by standard real-time qPCR (Applied Biosystems). 
Mir-33 was quantified using the TaqMan microRNA assay kit for has-miR-33a/b (Applied Biosystems), 
with U6 RNA used as an internal control.

Northern blot analysis. Poly A-tailed mRNA fractions were was separated in a 0.8% agarose gel 
containing 2% formaldehyde and transferred onto a nylon membrane Hybond-N+ (Amersham 
Biosciences). The membranes were hybridized with a 32P-labeled ABCA1 total and L3 cDNA probes 
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sense1 5’-AACCCAGGCACACUCUCUCUGUAGC-3’, antisense1 5’-AACCCAGGCACACUCUCUCUGUAGC-3’, 
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sense3 5’-UGGCGCGGCAAGUGCGCGGCUUUU-3’, antisense3 5’-UGGCGCGGCAAGUGCGCGGCUUUU-3’, 
sense4 5’-AAGGGGAGUGCGCUUUCUCCUGWCU-3’, antisense4 5’-AAGGGGAGUGCGCUUUCUCCUGWCU-3’, 

For microRNA assays, total RNA was extracted from cells using miRNA miRNA isolation 
kit (Ambion, Invitrogen) and reverse transcribed by standard real-time qPCR (Applied Biosystems). 
Mir-33 was quantified using the TaqMan microRNA assay kit for has-miR-33a/b (Applied Biosystems), 
with U6 RNA used as an internal control.
Western blot analysis. Cells were transiently transfected and treated as described in the figure legends. SDS-PAGE and Western blot analyses were performed as described previously. The immunoreactive proteins were visualized using ECL (GE Healthcare, Piscataway, NJ), and light emission was quantified with a LAS-3000 lumino-image analyzer (Fuji, Tokyo, Japan).

Cholesterol efflux assays. Cholesterol efflux was measured as previously described. In brief, JHH-5 cells were seeded into 12 well plates and transfected in triplicate with gene-specific Stealth RNAi or negative control Stealth RNAi. After 8 h transfection, the media was replaced with culture medium containing 0.5 µCi/ml [3H]-cholesterol and incubated for 24 h. Cells were then treated with 50 µM compactin with 40 µM mevalonate in medium containing 10% delipidated serum. After 16 h, cells were incubated with medium (2 mg/ml fatty acid-free BSA) with or without 10 µg/ml apoA-I for 6 h. The media was then collected and cleared of cellular debris by centrifuging at 800 x g for 10 min. The cell layers were dissolved in 0.1 N NaOH, and the percentage cholesterol efflux [media cpm/ (cpm media + cell associated cpm) x 100] was calculated by scintillation counting. Alternatively, free cholesterol mass released to the medium was determined enzymatically. In brief, cells were seeded into 6 well plates, and after 48 h transfection, the cells was incubated in medium containing 0.1%BSA with or without 10 µg/ml apoA-I for 24 h. Lipids released into the medium were extracted with chloroform/methanol (2:1, v/v), and free cholesterol mass was determined by highly sensitive Amplex Red cholesterol assay kit (Molecular Probes, Inc. OR, USA). The fluorescence intensity was measured using excitation at 530 nm and emission detection at 600 nm. The standard curve was linear between 0.05 and 2.0 nmol/assay.

Reporter gene assays. JHH-5 or HepG2 cells were transfected by the Effectene method using Effectene (QIAGEN) as recommended by the manufacturer or the Chen-Okayama method with luciferase reporter plasmid, expression plasmids, and empty vector. The total amount of transfected DNA was the same in each experiment. Luciferase activity was measured with a dual-luciferase reporter assay system (Promega, Madison, WI), and values were normalized to Renilla luciferase activity.

ChIP assay. ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology Inc., Lake Placid, NY) and the QIA quick PCR purification kit (QIAGEN) according to the manufacturer’s instructions. Briefly, JHH-5 cells were cross-linked with 1% formaldehyde and then washed with PBS. The cells were lysed on ice and the chromatin DNA was sheared by sonication into an average length between 0.2 and 1.0 kb fragments. The chromatin-protein fractions were incubated with anti-HNF4α or control IgG overnight at 4°C, followed by protein A beads. Chromatin immunocomplexes were washed and the DNAs were removed from the beads with the elution buffer and then heated along with DNA input samples overnight at 65°C to reverse the cross-links. Samples were then purified with QIA quick PCR purification kit (QIAGEN). Target regions were amplified by PCR using the following primers: human ABCA1 intron 3 sense (5’-TCCCTGAATGAAGAGCAGCTGACT-3’) and antisense (5’-AGGCAACACCCCAAAGCAAGGATGT-3’).

Avidin biotin-conjugated DNA (ABCD) assay. An oligodeoxynucleotide-avidin bead complex was prepared by mixing 100 pmol annealed biotinylated oligonucleotide with 10 µl avidin-beads (TetraLink™ Tetrameric Avidin Resin, Promega). The DNA-complex was mixed for 15 min and washed with TE buffer to eliminate unbound oligonucleotides. Cell lysates prepared from JHH-5 cells were pre-cleaned with avidin beads and then incubated with the DNA-bead complex for 30 min. After washing with lysis buffer, the samples were subjected to SDS-PAGE followed by Western blot. Biotinylated oligonucleotides used were as follows: ABCA1 intron 3 #1 sense (5’-TTAACCTGTGTTTTGAATTGCCCAGAGTCCTGCCACA-3’), antisense (5’-TTTGCAAGGACTCTGGCCTATTACACAGGTACATGGA-3’), ABCA1 intron 3 #2 sense (5’-TTTGCAAGGACTCTGGCCTATTACACAGGTACATGGA-3’), antisense (5’-TTTGCAAGGACTCTGGCCTATTACACAGGTACATGGA-3’).
**Electrophoretic mobility shift assay (EMSA).** Electrophoretic mobility shift assay was performed with the DIG Gel Shift Kit 2nd Generation (Roche Applied Science). The DIG-3’-end-labeled ABCA1 intron 3 intact (wild type) fragment (ttaactgtgtaaatgtctaaatggccagagtccc), its mutated fragment (ttaactgtgtaaatgtctaaatCggTTCgCAtcTctgcaga) or human apolipoprotein C-III (apoC-III) HNF4 binding site fragment (agctctcagcttgtactttggtacaactatgca) was incubated with the nuclear extracts of mock- or HNF4α-transfected 293 cells. In competition experiments, a 250-fold excess of unlabeled oligonucleotides, such as intact or mutated ABCA1 intron 3, was added to the reaction mixture. HNF4α-DNA complex was separated from free probe by 6% NOVEX retardation gel (Invitrogen) electrophoresis and blotted to Hybond-N+ (Amersham Biosciences). The shifted bands were visualized with AP-conjugated DIG antibody by using the chemiluminescence substrate.

**Lentivirus infection.** 293T cells were transfected with a lentiviral expression plasmid together with a packaging (pCAG-HIVgp) and a VSV-G- and Rev-expression plasmid (pCMV-VSV-G-RSV-Rev) by the calcium phosphate transfection method. After 24 h, cells were additionally cultured with a fresh medium containing 10 μM forskolin for 24 h. The medium containing lentviruses was collected and filtered. JHH-5 cells were infected with a medium supplemented with 10 μg/ml polybrene by a centrifugation method (2500 rpm for 90 min). Then the cells were refed with a fresh culture medium.

**Statistical analysis.** All results are representative of more than two separate experiments. Data were analyzed for statistical significance by Student t test or by ANOVA with the Student-Newman-Keuls test as a hoc test. Significance was established at the P < 0.05 level.

**Reference**


Fig. 1 (A) Structures of major human ABCA1 transcripts identified in this study. Variant-specific and universal probe/primers for quantitative real-time RT-PCR are indicated. The transcription start site of type P in the human genome ABCA1 is position 1. (B) Structure of rat and mouse liver type ABCA1 transcript.
Fig. I (C) Nucleotide sequence of 5'-region of the human ABCA1 gene. The sequences of universal and variant-specific exons are represented by large characters and by boxes. Amino acid residues encoded by exon 2, exon L3, exon 3 and exon 4 are indicated below the appropriate codons. A known translation start codon located on exon 2 and the predicted start codon located at the 3'-end of exon L3 are indicated by asterisk. The regulatory elements by transcription factors are underlined. The transcription start site of type P in the human genome ABCA1 is position 1. LXRE: LXR-response element; SRE: the region corresponding to rodent SREBP-response element.
Fig. I (D) Structures of minor human ABCA1 transcripts identified in this study.
**Fig. II**

Relative abundance of type L4a and L4b transcripts in different human tissues was determined by quantitative real-time RT-PCR using the specific probe/primer sets shown in Fig. IA. The multiple human tissue cDNA panel (BD MTC TM) in which expression levels were normalized represent the means ± S.D. (n=3) relative to expression levels in skeletal muscle.

**Fig. III**

The N-terminal amino acid residues corresponding type L3, type P, and type L2a/b ABCA1 mRNA. The predicted start codon for type L3 is located at 3'-end of exon L3. The type L3 ABCA1 protein lacks the amino-terminal 21-aa.
Fig. IV  Suppression of type L3 transcript expression by L3-specific siRNAs. JHH-5 cells were transfected with either siRNAs against type L3(#1-4) or a negative control siRNA. After 32 h, the cells were treated under sterol depleted conditions as in Fig. 2B for 16 h. The mRNA levels were measured by quantitative RT-PCR and normalized with 18S rRNA. The data represent the means ± S.D. (n=3).

Fig. V  Detection of L3 mRNA by Northern blot analysis. JHH-5 cells were transfected with either siRNA against ABCA1 type L3 (#1, #2), HNF4α (#1, #3) or a negative control siRNA. After 32 h, cells were treated with compactin (50 μM and 40 μM mevalonate: sterols -) for 16 h. Total poly A mRNA was prepared and analyzed by Northern blot using universal and L3-specific ABCA1 and GAPDH probes. The both ABCA1 mRNAs were detected at approximately 10 kb. Right half of this image is shown in Fig. 3.
Fig. VI  Nucleotide sequences of the promoter region of type L3 transcript and its cholesterol-responsive enhancer elements. The transcription start site of type L3 is indicated as +1. The exons are represented by boxes. P-box: a palindromic sequence box; HNF1: a putative HNF-1-binding site.
Fig. VII  Sequence alignment of five mammalian ABCA1 genes identified a highly conserved region in intron 3. The conserved sequences are represented by boxes. The 5'-end of each intron 3 is position 1. P-box: a palindromic sequence box; HNF1: a putative HNF1-binding site; HNF4: a putative HNF4-binding site.
Fig. VIII  Binding of nuclear HNF4α protein to the ABCA1 gene intron 3 was indicated by electrophoretic mobility shift assay (EMSA). (A) Probe sequences of ABCA1 intron 3 wild type (wt) and mutant (mut) and that of an HNF4-binding site in the apoC-III promoter (used as a positive control) are represented. (B) 293 cells were transiently transfected with mock- or HNF4α-expression vector, and then incubated for 36 h. Nuclear extracts were extracted and EMSAs were performed with Digoxigenin (DIG)-labeled probes. Specific bands presented in nuclear extract of HNF4α-transfected cells with an intron 3 wt probes and an HNF4-binding site probe. (C) Super-shift analysis and competition analysis were performed using anti-HNF4α antibodies (Ab) and unlabeled probes, respectively. Specific bands with HNF4α-nuclear extract were super-shifted in top on the gel (or deleted), indicating these complexes include HNF4α protein. In addition, EMSA were performed with DIG-labeled probes of ABCA1 intron 3 wild type and the nuclear extracts of HNF4α-transfected cells in the presence of a 250-fold excess of unlabeled probes of intron 3 wild type but not its mutant probe. Ab: antibodies, comp: Competitor, m: mock-transfected nuclear extract, H: HNF4α-transfected nuclear extract. Control IgG was used for supershift analysis.
Hep3B cells were transfected with either siRNA against HNF4α (#1, 3) or a negative control siRNA. After 32 h, the cells were treated with sterols (10 µg/ml cholesterol plus 1 µg/ml 25-hydroxycholesterol: sterols +) or compactin (50 µM, with 40 µM mevalnate: sterols -) in medium containing 10% delipidated serum for 16 h. (A) The mRNA levels of HNF4α and ABCA1 were measured by quantitative RT-PCR and normalized with 18S rRNA. The data represent the means ± S.D. (n=3). (B) The cells were harvested and the cell lysates analyzed by immunoblotting using anti-ABCA1, anti-HNF4α and anti-β-actin antibodies. (C) Hep3B cells were transfected with either siRNA against HNF4α (#1 and #3) or negative control siRNA. The cells were cultured under sterol-depleted conditions for 16 h (sterols -) and the cell lysates were analyzed by immunoblotting. Cholesterol efflux to the media was determined as described in Materials and Methods. The data represent the means ± S.D. *, p<0.05 (n=3) compared with control siRNA-transfected cells.
Fig. X  The effect of ectopic HNF4α expression on ABCA1 expression and cholesterol efflux in JHH-5 cells. JHH-5 cells were infected with empty (mock-infected) or HNF4α-expressing lentiviral vector and incubated for 48 h. (A) The cell lysates were analyzed by immunoblotting using anti-Flag and β-actin antibodies. (B) The mRNA levels were measured by quantitative RT-PCR and normalized with 18S rRNA. The data represent the means ± S.D. (n=3). (C) Cholesterol efflux to the media was determined as described in Materials and Methods. The data represent the means ± S.D. *, p<0.05 (n=5) compared with mock-transfected cells.
Fig. XI The effect of HNF4α siRNAs on LXRα, LXRβ and micro-RNA-33 (miR-33) expression in JHH-5 cells. JHH-5 cells were transfected with either siRNA against HNF4α (#1, 2, 3) or a negative control siRNA. After 32 h, the cells were treated with sterols (10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol: sterols +) or compactin (50 μM, with 40 μM mevalonate: sterols -) in medium containing 10% delipidated serum for 16 h. The mRNA levels of HNF4α, LXRα and LXRβ were measured by quantitative RT-PCR and normalized with 18S rRNA. MiR-33a level was normalized with U6 RNA. The data represent the means ± S.D. of three experiments. *, p<0.05 compared with control siRNA-transfected cells (Sterols +). #, p<0.05 compared with control siRNA-transfected cells (Sterols -).
Fig. XII  siRNA-mediated HNF4α knockdown reduced type L3 enhancer-promoter activity in a manner dependent on the HNF4α-binding site. JHH5-cells were transfected with the indicated reporter plasmids and control plasmid phRL-SV40 in the presence of either siRNA against HNF4α (#1) or a negative control siRNA. After 30 h, the cells were treated with sterols (10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol: sterols +) or compactin (50 μM, with 40 μM mevalonate: sterols -) for 16 h. The firefly luciferase activity in cell lysates was measured and normalized with Renilla luciferase activity. The data represent the means ± S.D. (n=3).

SV40: the SV40 promoter.
Fig. XIII Schematic diagram of the regulation of ABCA1 gene expression in human liver. Expression of the ABCA1 gene in human liver is regulated by a dual-promoter system. Under low cholesterol conditions, HNF4α activates the expression of liver-specific ABCA1 transcript including type L3 and L2b. Conversely, high cholesterol levels induce type P expression driven by the LXRE promoter. This dual promoter system ensures constant expression of hepatic ABCA1 under fluctuating cholesterol levels, which would play a role in maintaining plasma HDL levels. Note that the SRE promoter that drives liver-specific type L transcript expression in rodents is not functional in humans.
Fig. XIV  The SREBP binding element in human L2 promoter does not respond to sterols.
(A) Sequence alignment of SREBP binding elements in human and rodent ABCA1 L2 promoters.
(B) JHH-5 cells were transfected with the indicated reporter plasmids and control plasmid phRL-SV40. After 30 h, the cells were treated with sterols (10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol: sterols +) or compactin (50 μM, with 40 μM mevalonate: sterols -) for 16 h. The firefly luciferase activity in cell lysates was measured and normalized with Renilla luciferase activity. The data represent the means ± S.D. (n=3). The transcription start site of type L2 is indicated as +1. The SREBP binding site are represented by thick boxes.
Fig. XV  The effect of SREBP2 siRNAs on SREBP2, ABCA1, HNF4α and HMG-CoA Reductase mRNA expression. JHH-5 cells were transfected with either siRNA against SREBP2 (#1, 2, 3) or a negative control siRNA. After 32 h, the cells were treated with sterols (10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol: sterols +) or compactin (50 μM, with 40 μM mevalnate: sterols -) in medium containing 10% delipidated serum for 16 h. The mRNA levels of indicated genes were measured by quantitative RT-PCR and normalized with 18S rRNA. The data represent the means ± S.D. (n=3).
HDL 콜레스테롤 농도를 일정하게 관리하는 주요 기전은?:
HNF4α를 통한 간세포 ABCA1 조절

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Summary

배경
간세포의 ATP-binding cassette transporter A1 (ABCA1)은 혈중 HDL 콜레스테롤 농도 조절에 중요한 역할을 담당한다. 말초조직의 ABCA1은 세포 내 콜레스테롤을 방출하는 역할을 담당한다. 콜레스테롤 부족 상황이 되면 말초조직 ABCA1의 발현은 sterol regulatory element-binding protein-2 (SREBP-2) system에 의해 감소하지만, 간세포 ABCA1의 발현은 유지된다. 인체 간세포에서는 ABCA1 발현이 SREBP-2에 의해 크게 좌우되지 않는다고 보고되었다. 이 연구에서는 인체 간세포의 ABCA1 유전자 발현 조절의 주요 기전을 알아보았다.

방법 및 결과
ABCA1 mRNA L3형이 간세포 전체 ABCA1 mRNA의 25%를 차지하였고 세포의 콜레스테롤 부족 유도를 통해 발현이 유의하게 증가하였다. ABCA1 mRNA L3의 발현 억제 또는 발현 촉진은 콜레스테롤 역수송에 관여하는 ABCA1 발현과 기능을 결정하였다. 인체 ABCA1 유전자의 intron 3에 발현 조절 부위가 존재하였고, 콜레스테롤 부족에 의해 hepatocyte nuclear factor (HNF) 4α가 이 부위에 부착하여 작용하였다. HNF4α를 제거하면 인체 간세포의 ABCA1 mRNA L3 및 L2b형 발현이 감소하여 HDL 콜레스테롤 농도와 콜레스테롤 역수송 활성도가 감소하였다.

결론
HNF4α는 콜레스테롤 부족에 반응하여 인체 간세포 ABCA1 발현을 조절함으로써 혈중 HDL 콜레스테롤 농도 유지에 관여한다.
Commentary

ABCA1은 세포막에 존재하는 콜레스테롤과 인지질 수용체로서 apoA-I에 콜레스테롤과 인지질을 공급하여 HDL 콜레스테롤이 형성되도록 하는 중요한 역할을 담당한다. ABCA1 유전자 변이에 따른 HDL 콜레스테롤의 감소 또는 결핍은 Tangier병을 발생하며, 동물실험에서도 유전자 변이에 의해 ABCA1의 역할이 증명되었다.

대식세포나 섬유세포 등에 존재하는 말초조직의 ABCA1은 세포 내의 과다한 콜레스테롤을 apoA-I 혹은 디스크 모양의 초기 HDL 콜레스테롤에 전달하여 HDL 콜레스테롤을 구형으로 커지게 한다. 이렇게 형성된 HDL 콜레스테롤이 간으로 전달됨으로써 콜레스테롤 역수송이 이루어진다. 세포의 콜레스테롤 함량에 의해 활성도가 변하는 LXR 경로를 통해 말초조직 ABCA1 발현이 조절됨으로써 이에 따른 콜레스테롤 역수송 활성도가 결정되며, 이를 통해 세포 내의 콜레스테롤 함량이 일정하게 유지된다. 간세포 ABCA1 발현은 콜레스테롤 함량에 의해 크게 좌우되지 않으며 설치류의 SREBP-2와의 연관이 있다고 알려진 microRNA-33이 인체 간세포 ABCA1의 발현을 일정 부분 억제한다는 연구결과도 있다.1 HNF4α 결합 부위는 모든 포유동물에 존재하고 있기 때문에 다른 동물에서의 역할도 추가로 규명해야 할 부분이다.

결국 ABCA1 발현이 한가지 단독 인자에 의해 조절되는 것 보다는 여러 인자에 의해 복합조절을 받는 것이 타당한 추측이며, 항후 콜레스테롤 함량이 SREBP 혹은 HNF4α 등 여러 경로에 어떤 시그널을 통해 영향을 미치는가에 대한 연구도 흥미로운 결과를 알려줄 것으로 기대된다.

REFERENCES
HNF4α Increases Liver-Specific Human ATP-Binding Cassette Transporter A1 Expression and Cholesterol Efflux to Apolipoprotein A-I in Response to Cholesterol Depletion

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Objective—Hepatic ATP-binding cassette transporter A1 (ABCA1) plays the major role in maintaining plasma high-density lipoprotein levels by producing cholesterol-accepting nascent high-density lipoprotein, whereas peripheral ABCA1 is responsible for releasing cellular cholesterol. We previously reported that in rodents, cholesterol depletion reduces ABCA1 expression in peripheral but not hepatic cells by increasing a liver-specific ABCA1 transcript via the sterol regulatory element-binding protein-2 system. However, the regulatory element is not conserved in humans. Here we investigated the mechanism of sterol-regulated human hepatic ABCA1 gene expression.

Methods and Results—ABCA1 mRNA variant type L3 is a novel and human-liver-specific transcript accounting for ~25% of total ABCA1 mRNA in the liver and is induced by cellular cholesterol depletion. Specific knockdown or forced expression revealed that type L3 produces functional ABCA1 protein in cholesterol efflux. We identified a regulatory enhancer element for L3 expression lying within intron 3 of the human ABCA1 gene, to which hepatocyte nuclear factor (HNF) 4α binds in response to cholesterol depletion. HNF4α knockdown abolished induction of liver-specific L3 and L2b transcripts (and consequently the liver-type response of ABCA1 expression to cellular cholesterol status) and diminished cholesterol efflux activity.

Conclusion—These findings indicate that HNF4α regulates human hepatic ABCA1 expression in response to cholesterol depletion. (Arterioscler Thromb Vasc Biol. 2012;32:1005-1014.)

Key Words: ABC transporter • gene expression • lipoproteins • cholesterol regulation

The ATP-binding cassette transporter A1 (ABCA1) is a plasma membrane transporter involved in cholesterol and phospholipid transport that plays a critical role in apolipoprotein A-I (apoA-I)–dependent biogenesis of high-density lipoprotein (HDL). ABCA1 gene mutations cause Tangier disease and familial HDL deficiencies characterized by a near absence of circulating HDL. The essential role of ABCA1 in HDL biogenesis was further demonstrated by studies that included ABCA1 overexpression and deletion in mice.

Although ABCA1 is widely expressed in many tissues, gene-targeting studies in mice revealed that hepatic ABCA1 is responsible for approximately 80% of plasma HDL production. Further studies suggested that the role of hepatic ABCA1 in maintaining plasma HDL levels is distinct from that of extrahepatic ABCA1. Hepatic ABCA1 is critical for phospholipidating lipid-free apoA-I to generate cholesterol-poor early nascent HDL particles that in turn provide efficient cholesterol acceptors for peripheral cells, whereas ABCA1 in peripheral cells, such as macrophages and fibroblasts, mediates release of excess cellular cholesterol by generating mature HDL particles, probably in a sequential collaboration with ABCG1. The proposed roles of hepatic and peripheral ABCA1 are consistent with a classic model of the reverse cholesterol transport pathway, in which HDL transports excess cholesterol from peripheral cells to the liver, where cholesterol can be converted into bile acids for excretion.

Hepatic and peripheral ABCA1 are under distinct gene regulation. ABCA1 expression in peripheral cells is upregulated by a liver X receptor (LXR)–driven promoter system in response to cholesterol loading. Conversely, cholesterol depletion or treatment of cells with statins that inhibit endogenous LXR ligand synthesis represses ABCA1 expression, collectively indicating that cellular cholesterol homeostasis is stringently controlled by the LXR-ABCA1 pathway in peripheral cells. In contrast, hepatic ABCA1 expression appears to be unaffected by sterols as suggested by the failure of cholesterol feeding of mice to elevate hepatic ABCA1 expression and the inability of cholesterol depletion to repress expression. In the preceding study using rodents, we identified a liver-specific ABCA1 mRNA...
variant and a corresponding promoter element driven by sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor activated by cellular cholesterol depletion, in addition to the peripheral-type ABCA1 mRNA in the liver. Thus, cholesterol negatively and positively affects the liver-type SREBP-2 and peripheral-type LXR promoters, respectively, to control hepatic ABCA1 expression. This SREBP-2- and LXR-driven dual promoter system appears to ensure that hepatic ABCA1 expression is constant even under fluctuating cholesterol conditions and may be associated with ABCA1’s specific role in producing cholesterol acceptors in the reverse cholesterol transport pathway. Recently, microRNA-33 was shown to contribute to sterol-responsive ABCA1 gene regulation. MicroRNA-33, located within the gene encoding SREBPs, inhibits the expression of ABCA1 and makes a significant contribution to hepatic ABCA1 and plasma HDL levels in mice. Thus, multiple systems are likely to regulate hepatic ABCA1 expression.

In human hepatoma HepG2 cells, ABCA1 mRNA levels are slightly augmented on statin treatment, which suggests that a liver-type regulation also occurs in humans. However, the SREBP-2 binding element (sterol regulatory element) in the liver-type promoter is not conserved in humans, suggesting that a different system may drive liver-specific ABCA1 expression in humans. We report here that hepatocyte nuclear factor (HNF) 4α regulates expression of liver-specific ABCA1 transcripts in response to cholesterol depletion.

Methods
An expanded Material and Methods section is available in the online-only Data Supplement. 5′-rapid amplification of cDNA end polymerase chain reaction (PCR), plasmid construction, cell culture, RNA extraction and quantitative real-time reverse transcription (RT)-PCR, Northern and Western blot analyses, RNA interference, reporter gene assays, cholesterol efflux assays, chromatin immunoprecipitation assay, electrophoretic mobility shift assay, avidin biotin–conjugated DNA assay, lentivirus infection, and statistical analysis are described in detail in the online-only Data Supplement.

Results
Expression of Liver-Specific ABCA1 mRNA Variants in Human Liver Tissue and Hepatic Cells
To identify liver-specific ABCA1 transcripts, we performed 5′-rapid amplification of cDNA end containing 5 major ABCA1 mRNA 5′-ends, which were designated types L2a/b, L3, and L4a/b (Figure 1B, bottom; details are shown in Figure IA in the online-only Data Supplement), in addition to the previously described peripheral-type (type P) ABCA1 transcript. In contrast, peripheral THP-1 cells had only type P transcript. Type L3 is a novel transcript that lacks exon 1/exon 2 and has a newly identified 303-bp sequence (exon L3) upstream of exon 3. Exon L3 appears to be unique to humans because of no homologous sequence in rodent ABCA1 genes. Type L2a and L2b were homologous to rodent types L and L’, which we previously identified (Figure IA–IC in the online-only Data Supplement). Type L4a and L4b were similar to the human liver-type transcripts previously identified and were expressed mainly in the liver (Figure II in the online-only Data Supplement). Type L2b was less abundant compared with type L2a. Several minor transcripts, including those identified by Singaraja et al and Cavelier et al, are shown in Figure ID in the online-only Data Supplement. All products identified here were spliced according to the GT-AG splicing rule. Quantitative real-time RT-PCR (qRT-PCR) showed that newly identified type L3 and L2b variants were expressed exclusively in the liver, whereas type P was expressed in various human tissues (Figure 2A). Type L2a levels were not measured because there were no variant-specific regions in this transcript. To determine the abundance of type L3, L2b, or type P relative to that of total ABCA1 mRNA, we used variant-specific and universal RT-PCR probe/primers (Figure IA in the online-only Data Supplement) and a standard curve based on serial dilution of cDNAs containing 2 target sequences for the qRT-PCR probe (L3/universal, L2b/universal, or P/universal, respectively). The type L3 mRNA represented 26.2±9.4% of total ABCA1 mRNA in human liver RNA preparations (n=4), whereas type P and type L2b mRNA were estimated to be 33.4±5.5% and 12.3±4.8%, respectively, of total ABCA1 mRNA, indicating that type L3 is a novel and major ABCA1 transcript expressed in human liver. Type L3 was expressed in purified human hepatocytes at a higher level than that in the original total liver-cell preparations (Figure 2B).
Type L3 and L2b mRNAs were highly expressed in human hepatoma cells (HepG2, Hep3B, and JHH-5) at levels comparable to that in human liver tissues (n=4) but undetectable in peripheral THP-1 cells (Figure 2C). The expression of type L3 and L2b transcripts was augmented by cholesterol depletion in 3 hepatoma cell lines, as was rodent type L.\textsuperscript{14} Conversely, in all cell lines tested, type P mRNA expression decreased in response to cholesterol depletion, as was reported previously.\textsuperscript{9} Type L4a and L4b mRNA levels were extremely low and undetectable in all cell lines (data not shown). The total ABCA1 mRNA level in hepatic cell lines was either unaffected or mildly elevated by cholesterol depletion, whereas for peripheral THP-1 cells, total ABCA1 mRNA declined similarly to type P mRNA levels. These findings indicate that ABCA1 mRNA expression in human hepatoma cell lines is composed of sterol-repressed (type L2 and L3) and sterol-augmented (type P) mRNA expression.

Type L3 Is a Major ABCA1 Transcript Producing a Functional ABCA1 Protein
We investigated whether type L3 mRNA encodes a functional ABCA1 protein using a short interfering RNA (siRNA) specific to the type L3 transcript. In cholesterol-depleted JHH-5 and Hep3B cells, transfection of type L3-specific siRNA effectively diminished type L3 mRNA expression without decreasing type L2b and type P mRNA levels (Figure 3A). Notably, specific knockdown of type L3 mRNA caused a significant reduction in the total ABCA1 mRNA (Figure 3A) and protein levels (Figure 3B). To exclude off-target effects, we repeated the experiments using 3 other siRNAs specific to type L3, and we confirmed that these siRNAs also effectively diminished type L3 mRNA and decreased total ABCA1 mRNA and protein expression (Figure 3D and Figure IV). Northern blot analysis confirmed the L3 mRNA knockdown and an accompanying decrease in total ABCA1 mRNA band (Figure 3C). Furthermore, siRNA treatment caused an \( \approx 45\% \) decrease in apoa-I-dependent \(^{1}\H_{2}O\) cholesterol efflux from JHH-5 cells under cholesterol-depleted conditions (Figure 3E). Also, 3 siRNAs to type L3 significantly decreased the mass of free cholesterol efflux under normal culture conditions (Figure 3F). Although type L3 ABCA1 mRNA lacks exon 2, which includes a known translation start codon, ATG\textsuperscript{10,23} (Figure 1B and Figure III in the online-only Data Supplement), the L3 transcript could be initiated at using the predicted start codon located at the 3’-end of exon L3 (Figures IC and III in the online-only Data Supplement). We constructed an expression vector of type L3 ABCA1 fusing green fluorescent protein to the C terminus and obtained JHH-5 cells stably expressing L3-ABCA1–green fluorescent protein (Figure 3D and Figure IV).

Identification of the Type L3 Promoter and Its Cholesterol-Responsive Enhancer Elements
To explore the mechanisms by which type L3 transcripts were increased by cholesterol depletion, we searched for a possible promoter region for the L3 transcript in the region upstream of the putative transcription initiation site (+1) for type L3 mRNA (1010 or 4208 bp) (Figure 4A and 4B, \textit{Luc1–Luc3}; Figure VI in the online-only Data Supplement). Although these regions did not have basal promoter activity and were unresponsive to cholesterol depletion in a luciferase assay, a reporter con-
Figure 3. Specific knockdown or forced expression of type L3 mRNA modulates total ATP-binding cassette transporter A1 (ABCA1) mRNA and protein expression and apolipoprotein A-I (apoA-I)–dependent cholesterol efflux. A to F, JHH-5 or Hep3B cells were transfected with either short interfering RNA (siRNA) against ABCA1 type L3 (A, B, and E, #1; C to F, #1, 2, 3, or 4) or a negative control (ctrl, con) siRNA. After 32 hours, cells were treated with compactin (50 and 40 μmol/L mevalonate: sterols−) for 16 hours. A, Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) analysis of total and variant ABCA1 mRNA levels. Data were normalized with 18S rRNA and represent means±SD (n=3). C, Northern blot analysis of total and variant ABCA1 mRNA levels using ABCA1 total and L3-specific probes. B and D, Immunoblot analysis of ABCA1 and the loading control anti-β-actin. Bar graphs represent means±SD (n=4). E, F, and H, Efflux of [3H]cholesterol (E) or cholesterol mass (F and H) from the cells to the media was determined as described in Materials and Methods. G and H, JHH-5 cells stably expressing ABCA1 type L3–green fluorescent protein (GFP). G, Immunoblot analysis for ABCA1, GFP, and the loading control anti-β-actin. Bar graphs represent means±SD (n=3). H, Efflux of cholesterol mass from the cells to the media was determined. Data represent means±SD (n=3). *P<0.05 vs control siRNA-transfected (A-F) or mock-transfected (H) cells.

Struct containing an element from +426 to +480 did show basal transcriptional activity and caused mild activation on cholesterol depletion (Figure 4B, Luc5), albeit at a level that is likely insufficient to produce a full response to steroids.

The promoter activities of several genes are often regulated by distal enhancers. Sequence alignment of 5 mammalian ABCA1 genes identified a highly conserved region in intron 3 (positioned between +3103 and +3378 in humans) (Figure 4A; Figures VI and VII in the online-only Data Supplement). Therefore, we examined whether this region functions as an enhancer for the ABCA1 type L3 promoter by inserting the conserved region of intron 3 into the 3′-end of the poly A site (Figure 4A). We found that insertion of this region effectively enhanced type L3 promoter activity, and this activity was further amplified by cholesterol depletion (Figure 4C, Luc7). Furthermore, introducing this region into a reporter plasmid driven by the SV40 promoter instead of the type L3 promoter potentiated both basal promoter activity and a sterol response (Figure 4D, Luc9). These results suggest that the conserved region in ABCA1 intron 3 functions as a distal enhancer of the type L3 promoter that could be responsible for upregulation under sterol-depleted conditions.

To identify the sterol-responsive elements in this enhancer region, we performed a deletion analysis in this region. Deleting the 5′-end up to +3171 enhanced the promoter activity and sterol-mediated response (Luc10), whereas further deletion greatly diminished these effects (Figure 4E, Luc11–Luc14). The region between +3172 and +3183
contains a palindromic sequence box (P-Box) (Figure 4E, upper scheme). In a similar fashion, 3'-end deletions up to +3260 enhanced the promoter activity and the response to sterol depletion (Luc16), whereas deletion up to +3247 (Luc17) markedly decreased the response, and further deletion up to +3230 (Luc18) completely abolished these effects. Accordingly, a reporter containing the region from +3172 to +3259 showed a strong response to sterol depletion (Figure 4E, Luc19). Within this responsive region, deletion of either the 3'-end up to +3247 or the region between +3229 and +3241 significantly repressed the sterol-mediated response (Figure 4E, Luc20, Luc21). These results suggest that the P-box and the 2
elements (+3247 to +3259, +3229 to +3241) are required for the sterol-mediated response in the distal enhancer region in ABCA1 intron 3. A homology search detected consensus binding sequences for various transcription factors, suggesting that these elements (+3230/+3243 and +3224/+3256) could be binding sites for liver-enriched transcription factors such as HNF1 and HNF4, respectively (http://tfbind.ims.u-tokyo.ac.jp/; Figure 4E, upper scheme).

HNF4α Regulates Type L3 Promoter Activity Through Binding to the Distal Enhancer Elements

The role of liver-specific transcription factors HNF1α and HNF4α in the sterol-responsive distal enhancer activity within ABCA1 intron 3 was investigated. Whereas HNF4α expression effectively augmented type L3 promoter-enhancer activity in a distal enhancer region-dependent manner, HNF1α produced only minimal enhancement (Figure 5A). Expression of a dominant negative form of HNF4α lacking the AF-2 domain repressed its promoter-enhancing activity, especially under cholesterol-depleted conditions (Figure 5B). HNF4α expression also augmented the activity of a SV40 promoter-driven reporter gene containing the ABCA1 enhancer region (Figure 5C, Luc22) but did not activate a construct that lacked the putative HNF4α-binding site (Luc23). Deletion of the P-box (Luc24) or putative HNF1 site (Luc25) did not affect this HNF4α-mediated augmentation. In addition, the activity of a reporter gene containing 4 tandem repeats of the HNF4-response element was enhanced by HNF4α expression (Luc26). These results indicate that the HNF4-element, but not the P-box nor putative HNF1-binding site, is critical for HNF4α-mediated activation of ABCA1 expression.

Figure 5. Hepatocyte nuclear factor (HNF) 4α regulates ATP-binding cassette transporter A1 (ABCA1) type L3 promoter-enhancer activity by binding to the distal enhancer element. A to D, JHH-5 (A, B, and D) or HepG2 (C) cells were transfected with the indicated reporter plasmids and control plasmid control renilla luciferase plasmid-SV40 in the absence or presence of HNF4α or HNF1α expression vectors. A, C, and D, After 48 hours, the firefly luciferase (Luc) activity in cell lysates was measured and normalized with Renilla luciferase activity. B, After 30 hours of transfection, the cells were treated under sterols+ or sterols− conditions as in Figure 2C for 16 hours. DN indicates dominant negative. Data represent means±SD (n=5). SV40 indicates SV40 promoter. E and F, JHH-5 cells were treated under sterols+ or sterols− conditions for 16 hours. E, Cells were subjected to chromatin immunoprecipitation (IP) assay using the indicated antibodies or control IgG. The immunoprecipitated region containing HNF4α-response elements within the intron 3 enhancer region was amplified with polymerase chain reaction (PCR) using specific primers as described in Materials and Methods. The amplified PCR products were subjected to electrophoresis in a 2% agarose gel. Input indicates that genomes extracted from 2% of total lysates were amplified with PCR. F, The cell lysates were subjected to avidin biotin–conjugated DNA assays using 2 biotin-conjugated nucleotides (#1 or #2) as indicated in the top panel and, together with an aliquot of the lysates (input), immunoblotted (IB) with anti-HNF4α antibody. The arrow and asterisk indicate HNF4α protein and nonspecific bands, respectively.
We performed a chromatin immunoprecipitation assay to investigate whether endogenous HNF4α protein associates with chromatin via this putative HNF4-binding site. Clear bands were detected when chromatin from cholesterol-depleted JHH-5 cells was immunoprecipitated with antibodies against HNF4α, indicating that a tight association of endogenous HNF4α protein with the conserved intron 3 region was induced by cholesterol depletion (Figure 5E). Additionally, an in vitro DNA binding assay confirmed the association of HNF4α with this site. Endogenous HNF4α in lysates of cholesterol-depleted cells reacted with biotin-conjugated oligodeoxynucleotides corresponding to the \textit{ABCA1} region from \textasciitilde3223 to \textasciitilde3262 that includes the HNF4-response element (#1), but not oligonucleotides that lacked this element (#2) (Figure 5F). Furthermore, electrophoretic mobility shift assay also confirmed the association of HNF4α with this binding site (Figure VIII in the online-only Data Supplement). These results indicate that the putative HNF4-binding site on \textit{ABCA1} intron 3 is authentic.

HNF4α Is Required for Expression of Liver-Specific \textit{ABCA1} mRNA and Protein in Response to Sterol Depletion

The role of HNF4α in liver-type \textit{ABCA1} mRNA variant expression was investigated using siRNA knockdown. In JHH-5 cells, 3 siRNAs against HNF4α effectively reduced HNF4α mRNA and protein expression (Figure 6A and 6B). HNF4α knockdown resulted in the reduced expression of...
type L3 and also type L2b but not type P transcripts under sterol-depleted conditions (Figure 6A, black bars). Consistently, expression of total ABCA1 mRNA and protein was reduced by HNF4\(\alpha\) knockdown under sterol-depleted conditions (Figure 6A and 6B). Reduction of type L3 and total ABCA1 mRNA was also confirmed by Northern blot (Figure V in the online-only Data Supplement). In the presence of cholesterol, however, HNF4\(\alpha\) knockdown did not affect the expression of ABCA1 transcripts (Figure 6A, white bars), although 1 siRNA (#1) induced type P and total ABCA1 mRNA expression probably because of an off-target effect. Similar results were obtained with another human hepatic cell line Hep3B (Figure IXA and IXB in the online-only Data Supplement). In agreement with these results, HNF4\(\alpha\) knockdown in JHH-5 and Hep3B cells reduced apoA-I-mediated cholesterol efflux under cholesterol-depleted conditions (Figure 6C; Figure IXC in the online-only Data Supplement). Conversely, forced expression of HNF4\(\alpha\) increased the level of type L3 and L2b transcripts, leading to a significant increase in total ABCA1 mRNA expression and apoA-I-mediated cholesterol efflux (Figure X in the online-only Data Supplement). We also confirmed that HNF4\(\alpha\) knockdown markedly reduced the type L3 promoter-enhancer activity (Figure XIIA in the online-only Data Supplement) and the HNF4\(\beta\)-binding site-dependent intron 3 enhancer activity (Figure XIIIB in the online-only Data Supplement) in sterol-depleted cells but did not efficiently modulate the expression of LXRa, LXRB, and micro-RNA-33, which are regulatory genes of ABCA1 transcript (Figure XI in the online-only Data Supplement). These results indicate that HNF4\(\alpha\) is required for liver-specific ABCA1 transcript (L3 and L2b) expression and liver-type response of ABCA1 mRNA and protein expression on cholesterol depletion.

**Discussion**

The liver is the major source of apoA-I, and hepatic ABCA1 is responsible for producing the majority of plasma HDL. Hepatic ABCA1 has a specific role in producing early nascent HDL particles, and its genetic regulation is distinct from extrahepatic ABCA1. In this study, we investigated the regulation of ABCA1 expression in human liver cells and discovered a liver-specific regulatory system in humans. Among the liver-specific variants (types L2a/b, L3, L4a/b), type L3 is a novel ABCA1 transcript containing a human-specific exon L3 and is a major transcript accounting for \(\approx 25\%\) of total ABCA1 mRNA in human liver.

The ABCA1 protein translated from type L3 transcript lacks the amino-terminal 21 amino acids, because the first ATG codon resides in the 3\'-end of exon L3 (Figure III in the online-only Data Supplement). Although early studies showed that an ABCA1 expression construct lacking the 60 amino-terminal amino acids does not produce a functional protein in cells, specific siRNAs against type L3 efficiently reduced ABCA1 protein and cholesterol efflux. Moreover, transfection of L3-ABCA1 expression vector into JHH-5 cells augmented ABCA1 protein and cholesterol efflux activity, demonstrating that the L3-derived ABCA1 variant protein is stable and functionally active.

**HNF4\(\alpha\) Regulates Hepatic ABCA1 Gene Expression in Humans**

When cellular cholesterol is depleted, the liver-specific type L3 and L2b transcripts are induced and positively regulate ABCA1 protein expression (Figure XIII in the online-only Data Supplement). We previously discovered that SREBP-2, a transcription factor activated on cellular cholesterol depletion, upregulates the rodent liver-type (type L) ABCA1 promoter. However, the SREBP-2 binding element (sterol regulatory element) is in the rat promoter and is conserved among mammals (Figure XIVA in the online-only Data Supplement), and the human type L2 promoter containing this region is not activated by cholesterol depletion (Figure XIVB in the online-only Data Supplement). In humans, we found a critical role of the liver-enriched transcription factor HNF4\(\alpha\) in hepatic ABCA1 expression. Knockdown of HNF4\(\alpha\) abolished inductions of L3 and L2b mRNA and, conversely, forced expression of HNF4\(\alpha\) in JHH-5 cells increased them. Altered L3 and L2b mRNA levels led to substantial changes in total ABCA1 mRNA and protein expression and in cholesterol efflux activity. Thus, HNF4\(\alpha\) is required for the induction of liver-specific ABCA1 transcripts, including types L3 and L2b, and thereby modulates ABCA1 expression and HDL generation.

We identified a regulatory enhancer element for L3 expression lying within intron 3 of the human ABCA1 gene, to which HNF4\(\alpha\) binds and at which HNF4\(\alpha\) augments the L3 promoter-enhancer activity in response to cholesterol depletion. Because HNF4\(\alpha\) expression was increased on cholesterol depletion and HNF4\(\alpha\) knockdown abolished the L3 and L2b induction, it is likely that the HNF4\(\alpha\) level or activity (via dephosphorylation) determine the sterol-responsive L3 and L2b expression. However, it is more likely that along with HNF4\(\alpha\), several transcription factor(s) cooperatively regulate the L2 and L3 expression, because we identified several elements (and region) responding to cholesterol depletion in the intron 3-enhancer and the L3 promoter (Figure 4E). In addition, in human JHH-5 cells, SREBP-2 knockdown partially diminished the L3 and L2b induction on sterol depletion (Figure XV in the online-only Data Supplement). Thus, SREBP2 partially regulates human hepatic ABCA1 expression through distinct mechanism from that in rodents. Although decreased HNF4\(\alpha\) expression (Figure XV in the online-only Data Supplement) may be responsible for this effect, it is possible that SREBP-2 directly (via some unknown sterol regulatory element) or indirectly (via interaction with HNF4\(\alpha\)) regulates L3 and L2b mRNA expression. Decreased type P mRNA expression by SREBP-2 knockdown is probably due to the decreased supply of endogenous ligands for LXR.

HNF4\(\alpha\) knockdown resulted in the reduced expression of types L3 and L2b but not type P. Although we have confirmed that neither LXR regulating type P promoter nor microRNA-33 regulating ABCA1 mRNA stability was affected by HNF4\(\alpha\) knockdown, further investigation on chromatin-loop or transcription factors is needed to understand the mechanism.

Because the HNF4 binding site located in ABCA1 intron 3 is conserved among mammals (Figure III in the online-only Data Supplement), HNF4\(\alpha\) may also regulate the liver-type
ABCA1 transcript(s) in rodents. However, we could not confirm this because of the unavailability of variant-specific primers for rodent type L mRNA that corresponds to human type L2a. In addition, a rodent variant corresponding to human type L3 may not be expressed in rodent liver because exon L3 is not conserved in rodents. Studies have shown that acute loss of HNF4α (for 6–7 days) in mouse liver reduced ABCA1 mRNA expression, although Cre-loxP knockdown did not affect the level. Thus, the detailed mechanisms that regulate liver-specific ABCA1 gene expression may differ between humans and rodents. However, hepatic ABCA1 gene expression is ensured by stimulating liver-specific systems on cholesterol depletion, a response that is conserved among humans, mice, and rats.

HNF4α is a highly conserved member of the nuclear receptor superfamily. Loss-of-function mutations in the human HNF4α gene cause the disorder maturity onset diabetes of the young (MODY1). HNF4α is required for early liver development and expression of many liver-specific genes, including those involved in lipoprotein and cholesterol/bile acid metabolism. For example, HNF4α regulates expression of microsomal triglyceride transfer protein, apolipoprotein B, C, and E, genes involved in very-low-density lipoprotein production; cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme of bile acid biosynthesis from cholesterol; and ABCG5/ABCG8, transporters involved in cholesterol/sterol excretion from the liver. Together with these genes, HNF4α-regulated ABCA1 gene expression may cooperatively control hepatic cholesterol elimination to maintain cholesterol homeostasis. A recent study has shown that HNF4α-siRNA knockdown in mouse liver reduces expression of many lipid metabolism genes, including ABCA1, leading to decreases in HDL and very-low-density lipoprotein in plasma.

**Dual System Regulates Human Hepatic ABCA1 Gene Expression in Response to Sterols**

In the model of reverse cholesterol transport pathway, the liver plays a role in excreting cholesterol transported from peripheral tissues. The liver is also the major site of production of HDL, and hepatic ABCA1 is responsible for maintaining plasma HDL by producing a precursor form of HDL, which could accept cholesterol from peripheral cells. When cholesterol accumulates in the liver, the type P ABCA1 transcript is induced by the LXR promoter. However, this rise is counterbalanced by the reduced expression of liver-type L3 and L2a/b transcripts (Figure XIII in the online-only Data Supplement). The dual system may prevent overshooting ABCA1 expression and retransport of cholesterol to peripheral tissues. On cholesterol depletion, type P ABCA1 expression is reduced, but enhanced type L3 and L2a/b expression may compensate for this reduction. Thus, the dual system ensures constant expression of ABCA1 and HDL production in the human liver even under fluctuating cholesterol conditions. Recent studies show that microRNA-33 regulates hepatic ABCA1 expression and plasma HDL levels in mice. ABCA1 expression also undergoes posttranslational regulation. Because hepatic ABCA1 plays the major role in maintaining plasma HDL levels, it seems reasonable that hepatic ABCA1 expression would be stringently regulated by multiple sterol-responsive systems.

**Regulation of Human Hepatic ABCA1 as a Novel Strategy to Prevent Atherosclerosis**

Statins are widely used to treat hypercholesterolemia and raise plasma HDL cholesterol independently of reducing low-density lipoprotein cholesterol. Several studies have shown that statins upregulate hepatic ABCA1 mRNA expression in human hepatoma cells and mouse liver, whereas statins repress peripheral ABCA1 mRNA expression by depleting endogenous LXR ligands. Our findings indicate that the liver-specific ABCA1 variants, including type L3 and L2a/b (or L in rodents), are responsible for statin-induced ABCA1 expression and may be associated with increased plasma HDL levels.

Our current study clarified for the first time a mechanism by which human hepatic ABCA1 expression is regulated. HDL cholesterol levels are inversely correlated with cardiovascular risk. Because hepatic ABCA1 has the largest impact on plasma HDL levels and is the most promising therapeutic target, our findings provide a basis for developing novel drugs to control plasma HDL levels and prevent atherosclerosis.

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**Disclosures**

None.

**References**


