Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1

Cheryl L. Wellington,* Liam R. Brunham,* Steven Zhou,* Roshni R. Singaraja,* Henk Visscher,* Allison Gelfer,* Colin Ross,* Erick James,* Guoqing Liu,* Mary T. Huber,† Yu-Zhou Yang,* Robin J. Parks,§ Albert Groen, Jamila Fruchart-Najib,†† and Michael R. Hayden[1],*,†**

Centre for Molecular Medicine and Therapeutics,* British Columbia Children's and Women's Hospital, University of British Columbia, Vancouver, BC, Canada; Xenon Genetics, Inc.,† Vancouver, BC, Canada; Molecular Medicine Program,§ Ottawa Health Research Institute, Ottawa, ON, Canada; Academic Medical Centre,** Amsterdam, the Netherlands; and Institut Pasteur,†† Lille, France

Abstract **ATP binding cassette transporter A1 (ABCA1) is a widely expressed lipid transporter essential for the generation of HDL. ABCA1 is particularly abundant in the liver, suggesting that the liver may play a major role in HDL homeostasis. To determine how hepatic ABCA1 affects plasma HDL cholesterol levels, we treated mice with an adenovirus (Ad)-expressing human ABCA1 under the control of the cytomegalovirus promoter. Treated mice showed a dose-dependent increase in hepatic ABCA1 protein, ranging from 1.2-fold to 8.3-fold using doses from 5×10^8 to 1.5×10^9 pfu, with maximal expression observed on Day 3 posttreatment. A selective increase in HDL cholesterol occurred at Day 3 in mice treated with 5×10^8 pfu Ad-ABCA1, but higher doses did not further elevate HDL cholesterol levels. In contrast, total cholesterol, triglycerides, phospholipids, non-HDL cholesterol, and apolipoprotein B levels all increased in a dose-dependent manner, suggesting that excessive overexpression of hepatic ABCA1 in the absence of its normal regulatory sequences altered total lipid homeostasis. At comparable expression levels, bacterial artificial chromosome transgenic mice, which express ABCA1 under the control of its endogenous regulatory sequences, showed a greater and more specific increase in HDL cholesterol than Ad-ABCA1-treated mice. Our results suggest that appropriate regulation of ABCA1 is critical for a selective increase in HDL cholesterol levels.**—Wellington, C. L., L. R. Brunham, S. Zhou, R. R. Singaraja, H. Visscher, A. Gelfer, C. Ross, E. James, G. Liu, M. T. Huber, Y-Z. Yang, R. J. Parks, A. Groen, J. Fruchart-Najib, and M. R. Hayden. **Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1.** ***J. Lipid Res.*** **2003.** 44: **1470–1480.**

Supplementary key words ATP binding cassette transporter A1 • HDL cholesterol • adenovirus • bacterial artificial chromosome transgenic • hepatocytes • regulation

ATP binding cassette transporter A1 (ABCA1) is essential for the transfer of phospholipid (PL) and cholesterol to lipid-free apolipoprotein A-I (apoA-I) to form pre-β-HDL (1–3). This is the first step in reverse cholesterol transport (RCT), whereby excess cholesterol is removed from cells and transported as HDL to the liver for eventual excretion in bile (4–8). Absence of ABCA1 activity results in Tangier disease, which is characterized by a nearly complete loss of circulating HDL, an accumulation of cholesterol ester (particularly in cells of the reticuloendothelial system), and an increased risk of coronary artery disease (9–14).

Since the discovery of a highly significant inverse relationship between HDL levels and coronary artery disease, the metabolic origin of HDL has been a subject of intense investigation. The discovery that ABCA1 is essential for HDL biosynthesis has provided a unique opportunity to investigate RCT in detail. The prior hypothesis of RCT proposes that the majority of plasma HDL originates from peripheral tissues (4–8). However, ABCA1 is abundantly expressed in the liver compared with other tissues that participate in RCT (15, 16), suggesting that hepatocytes may themselves may play a major role in HDL biosynthesis. This is supported by the observation that hepatocytes are the site of the greatest accumulation of cholesteryl ester (CE) in the Wisconsin hypo-alpha mutant chicken, a naturally occurring animal model of ABCA1 deficiency (17). Furthermore, reconstitution of ABCA1-deficient mice with bone marrow from wild-type mice does not result in restoration of plasma HDL cholesterol levels, suggesting that monocytes and macrophages contribute only marginally to HDL cholesterol levels in vivo (18). Finally, hepatocytes are centrally involved in lipid homeostasis, and the mass of the liver relative to other organs is sufficient to make a major contribution to plasma HDL cholesterol.

These observations suggested that manipulation of ABCA1 in the liver may be a promising therapeutic strategy aimed at increasing plasma HDL cholesterol levels

Manuscript received 11 March 2003 and in revised form 30 April 2003.

Published, JLR Papers in Press, May 1, 2003.
DOI 10.1194/jlr.M300110-JLR200

[1] To whom correspondence should be addressed.
e-mail: mrh@cmmt.ubc.ca

and protecting from atherosclerosis. However, three crucial interrelated questions remain to be addressed. First, it is important to determine if there may be an optimal level of hepatic ABCA1 expression that results in a selective increase in plasma HDL cholesterol. Second, because overall lipid homeostasis is significantly coordinated by the liver, it is important to understand not only how the regulation of ABCA1 in the liver affects lipid efflux and formation of HDL particles, but also how hepatic ABCA1 may affect overall plasma lipids. Finally, it is essential to establish how HDL cholesterol levels may be influenced by ABCA1 in extrahepatic tissues such as the intestine.

We recently used transgenic technologies to produce bacterial artificial chromosome (BAC) transgenic mice expressing human ABCA1 (19). Importantly, the use of a BAC ensures that endogenous regulatory elements within the ABCA1 transgene are present and that ABCA1 is expressed with normal temporal-, tissue-, and cell-specific patterns. We have shown that activation of liver X receptor response elements by oxysterols in vivo directly contributes to an increase in human-specific ABCA1 mRNA and leads to increased expression of the human ABCA1 protein in an identical pattern as the endogenous gene (19). This appropriate regulation of ABCA1 results in a 1.6-fold increase in plasma HDL cholesterol levels without affecting other lipoprotein classes (19). Additionally, by crossing our BAC transgenic mice onto the well-established apoE$^{-/-}$ model of atherosclerosis, we have demonstrated that increased ABCA1 activity is associated with markedly reduced lesion size, as well as the appearance of less-complex lesions that lack fibrous caps and exhibit reduced foam cell involvement (20). These studies validate the BAC transgenic model as an excellent research tool for comparing the impact of increased ABCA1 expression due to endogenous versus other promoters on plasma lipid levels.

In order to evaluate how hepatic ABCA1 expression in the absence of its appropriate regulatory signals affects plasma HDL cholesterol levels, we developed an adenovirus (Ad)-expressing full-length human ABCA1 under the control of the cytomegalovirus (CMV) promoter (Ad-ABCA1). This vector allowed exogenous ABCA1 to be specifically overexpressed in the liver such that it is uncoupled from its normal regulatory circuits, and these results could be compared with results of ABCA1 overexpression in the BAC transgenic mice. In addition, mice could be treated with various doses of Ad-ABCA1 in order to determine how increasing the hepatic dosage of ABCA1 affected plasma HDL cholesterol levels. Our results suggest that the abundance and regulation of ABCA1 in the liver and extrahepatic tissues are crucial parameters in the regulation of plasma HDL cholesterol levels in vivo.

MATERIALS AND METHODS

Recombinant adenoviral vectors

The full-length human ABCA1 cDNA was subcloned into an E1/E3-deleted recombinant adenovirus (Ad5) vector backbone containing the CMV promoter-enhancer to generate Ad-ABCA1. Plaques positive for ABCA1 expression were identified and used for large-scale amplification in 293 cells grown in 150 mm culture dishes. Similarly, a recombinant Ad5 vector carrying the reporter alkaline phosphatase gene [Ad-alkaline phosphatase (AP) (21)] was used for concurrent control infections. Purification of these recombinant adenoviral vectors was performed by two sequential rounds of CsCl density gradient ultracentrifugation. The purified virus stocks were desalted over a Sephadex G-25 column (Amersham Pharmacia) and eluted in sterile HEPES buffered saline. Glycerol (15%) was added and the viral stock was stored at −80°C until use. Infectious viral titres were determined using the Adeno-X Rapid Titer Kit (Clontech), as suggested by the manufacturer. The replication incompetence of the E1/E3-deleted recombinant virus was verified by the lack of cytopathic effect after infection of COS-1 cells, which, unlike 293 cells, do not complement the gene deletion.

Cell culture

HeLa and HepG2 cells were cultured in DMEM high glucose (Canadian Life Technologies) supplemented with 10% fetal calf serum, 50 U/ml penicillin-streptomycin, and 2 mmol glutamine (Canadian Life Technologies). For in vitro infection, cells were seeded in 24-well dishes at a density of 20,000 cells/well. After 16 h, the conditioned media was removed and cells were incubated with various dilutions of stock virus in DMEM with 2% FBS for 1 h at 37°C, after which the conditioned media was replaced. After an additional 16–24 h, cells were used for efflux assays or Western analysis.

Animals and in vivo delivery of recombinant Ad

Male wild-type C57Bl/6 mice obtained from Jackson Laboratories were maintained on a standard chow diet (PMI Feeds). Adenoviral vectors were diluted in sterile PBS to deliver doses of 5×10^8 pfu/mouse, 7.5×10^8 pfu/mouse, 1×10^9 pfu/mouse, and 1.5×10^9 pfu/mouse for Ad-ABCA1, and 1.5×10^9 pfu/mouse for Ad-AP. The dose of Ad-AP was matched to the maximum dose of Ad-ABCA1 in order to control for any adverse effects due to high-dose Ad infection in vivo. A total of 200 μl of diluted virus was injected into the tail vein of immobilized mice. Additional mice received 200 μl of PBS only as a vehicle control. Preinjection blood samples were collected from the saphenous vein. After 3 or 7 days posttreatment, animals were sacrificed by CO_2 inhalation, blood was collected by cardiac puncture, and liver, spleen, and small intestine were isolated and immediately frozen at −80°C. BAC transgenic and wild-type control mice were maintained on chow or atherogenic diets as previously described (19). All procedures involving experimental animals were performed in accordance with protocols from the Canadian Council of Animal Care and the University of British Columbia Animal Care Committees.

Western blot and immunohistochemistry

Tissues were homogenized as previously described (22), and protein concentration was determined by Lowry assay. Equal amounts of protein were electrophoresed through 7.5% SDS polyacrylamide gels, electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore), and immunodetected using a monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2 of ABCA1) or anti-GAPDH (Chemicon) as a loading control (22). Blots were developed using enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations. Bands were quantitated by densitometry using NIH Image 6.1. In each lane, ABCA1 levels were normalized to GAPDH levels to control internally for protein loading. Data represent the mean and standard

deviation from tissue from three or four animals, each run at least in duplicate.

For histochemical analysis, tissues were fixed for 16 h in 4% paraformaldehyde in PBS prior to paraffin embedding. Hematoxylin-eosin and Giemsa staining were performed according to standard procedures by the University of British Columbia Histology Core Service.

RNA analysis

RNA from mouse liver was extracted using Trizol (Canadian Life Technologies) and purified using RNeasy reagents (Clontech), as recommended by the manufacturer. The relative levels of human and murine ABCA1 were determined by semiquantitative RT-PCR from 3 μg of total RNA as described (15).

Cholesterol and phosphotidylcholine efflux

Ad-ABCA1 or mock-infected HeLa cells and fibroblasts were labeled with 1 μCi/ml of [^{3}H]cholesterol (New England Nuclear) or [^{3}H]choline (Amersham) for 16 h. Labeled cells were then washed and equilibrated in DMEM with 0.2% BSA for 1 h, after which 20 μM of lipid-free apoA-I (Calbiochem) was added for 4 h. Efflux assays were performed as described previously (23). For cholesterol efflux, media and cell lysates were mixed with scintillant and counted directly (23). For phosphatidylcholine efflux, lipids from the media and cell lysates were first extracted with chloroform-methanol (2:1, v/v) prior to detection (24). Data are representative of the mean and standard deviations from at least two independent experiments, each performed in triplicate.

Analysis of bile and fecal sterol levels

Gall bladder bile was aspirated at sacrifice using a 30 G needle and frozen immediately at −80°C until use. Feces generated over a 24 h period were collected pre- and postinjection and stored at −80°C until use. Biliary bile salt and cholesterol concentrations were determined as previously described (25). Neutral and acidic fecal sterol content was determined by gas liquid chromatographic analysis as described (25).

Plasma lipid and lipoprotein analysis

All blood samples were collected following a 4 h fast. After centrifugation for 10 min at 12,000 rpm at 4°C, plasma was frozen at −80°C until use. Serum and lipoprotein lipid [total cholesterol (TC), triglycerides (TGs), and PLs] concentrations were determined by enzymatic assays adapted to microtiter plates using commercially available reagents (Boehringer Mannheim, Germany) as previously described (20). Serum HDL cholesterol levels were determined after precipitation of apoB-containing lipoproteins with phosphotungstic acid/mg (Roche Diagnostics GmbH, Mannheim). Serum levels of apoB were measured by an immunonephelemetric assay using a specific mouse polyclonal antibody as previously described (20). Tabulated data represent the means and standard deviations of three or four mice per group. Relative changes in Day 3 plasma lipids in Ad-ABCA1- or Ad-AP-treated mice were determined by normalizing values from treated animals to PBS-only controls. Data were then expressed as the percent change relative to the baseline observed in PBS-treated mice. Similarly, relative changes in plasma lipids in BAC transgenic mice were determined by normalized values in BAC transgenic mice on either a chow or atherogenic diet (19) to those observed in nontransgenic controls on the same diet. Data were then expressed as percent change relative to the baseline in wild-type animals. Data represent the means and standard deviations from at least four BAC or four wild-type mice. Statistical analysis was by one-way ANOVA with a Neuman-Keuls posttest using Graph-Pad Prism 3.03.

RESULTS

Adenoviral delivery of human ABCA1 promotes cholesterol and PL efflux in cultured cells

Expression and physiological activity of the Ad-ABCA1 was first confirmed in vitro. HeLa cells express very little endogenous ABCA1 and have a correspondingly low level of basal cholesterol or phosphatidylcholine efflux. HeLa cells infected with Ad-ABCA1 at a multiplicity of infection of 25 demonstrated enhanced ABCA1 expression as well as cholesterol and phosphatidylcholine efflux (**Fig. 1A**). Additionally, Ad-ABCA1 promoted a dose-dependent increase in cholesterol efflux in HepG2 cells (Fig. 1B).

Human ABCA1 is expressed specifically in liver following injection of Ad-ABCA1

Having validated the functional activity of Ad-ABCA1, male wild-type C57Bl/6 mice received doses of Ad-ABCA1

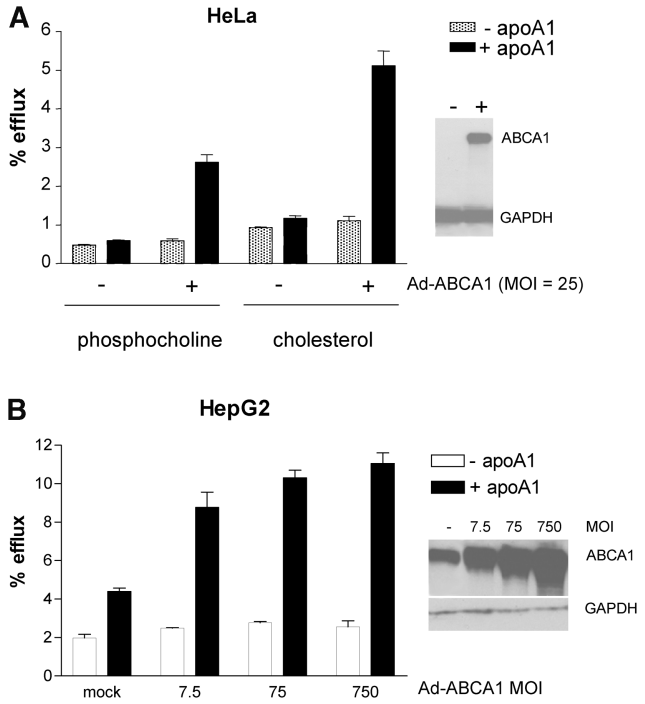

Fig. 1. Functional activity of adenovirus (Ad)-ATP binding cassette transporter A1 (ABCA1) in cultured cells. A: Apolipoprotein A-I (apoA-I)-dependent cholesterol and phosphatidylcholine efflux are specifically increased in HeLa cells infected with Ad-ABCA1. Cells were either mock infected (−) or infected with Ad-ABCA1 at a multiplicity of infection (MOI) of 25 (+), labeled with [^{3}H]cholesterol or [^{3}H]choline, and exposed to 20 μg/ml lipid-free apoA-I (black bars) or left untreated (stippled bars) for 4 h. Media and cells were collected separately and used to determine percent efflux. Data represent the mean and standard deviations of two independent, pooled experiments, each performed in triplicate. Western blot analysis (inset) demonstrates high expression of ABCA1 only in infected cells. B: Dose-dependent increase in cholesterol efflux in HepG2 cells by Ad-ABCA1. Cells were either mock infected (−) or infected with Ad-ABCA1 at MOIs of 7.5, 75, and 750, labeled with [^{3}H]cholesterol, and exposed to 20 μg/ml lipid-free apoA-I (black bars) or left untreated (clear bars) for 4 h. Media and cells were collected separately and used to determine percent efflux. Data represent the mean and standard deviations of triplicate measurements. Western blot analysis (inset) demonstrates high expression of ABCA1 only in infected cells.

ranging from 5.0×10^8 to 1.5×10^9 pfu/mouse, Ad-AP at 1.5×10^9 pfu/mouse, or PBS as a vehicle-only control, by tail vein injection. Liver, spleen, and small intestine were harvested 3 or 7 days after injection, and probed with an ABCA1-specific antibody to monitor expression levels in multiple tissues. As expected with intravenous delivery, exogenous ABCA1 expression was observed only in the liver (26), which exhibited a dose-dependent response of ABCA1 expression (**Fig. 2**). For example, an 8.3-fold increase in ABCA1 protein levels was observed in animals injected with the highest dose of Ad-ABCA1 (1.5×10^9 pfu/mouse) at 3 days, which declined by ~40% by Day 7 (Fig. 2A). No increase in ABCA1 expression was observed in spleen (Fig. 2) or small intestine (data not shown). Furthermore, RT-PCR analysis of RNA extracted from livers of Ad-ABCA1-injected mice confirmed that the increase in ABCA1 protein levels resulted from expression of exogenous human ABCA1 and not by up-regulation of endogenous murine ABCA1 (data not shown). These results demonstrated that ABCA1 abundance increased in a dose-dependent manner specifically in the livers of Ad-ABCA1-infected mice.

Plasma HDL cholesterol levels are elevated in Ad-ABCA1-treated mice, but not in a dose-dependent manner

As expected, HDL cholesterol levels were increased in Ad-ABCA1-infected mice compared with both PBS and Ad-AP controls, and maximal elevation of HDL cholesterol was typically observed at Day 3 in all Ad-ABCA1-treated mice (**Table 1**). By Day 7, HDL cholesterol levels tended to decrease, as expected, due to the decline in ABCA1 expression in the liver at that time (Fig. 2). Mice receiving the lowest dose of

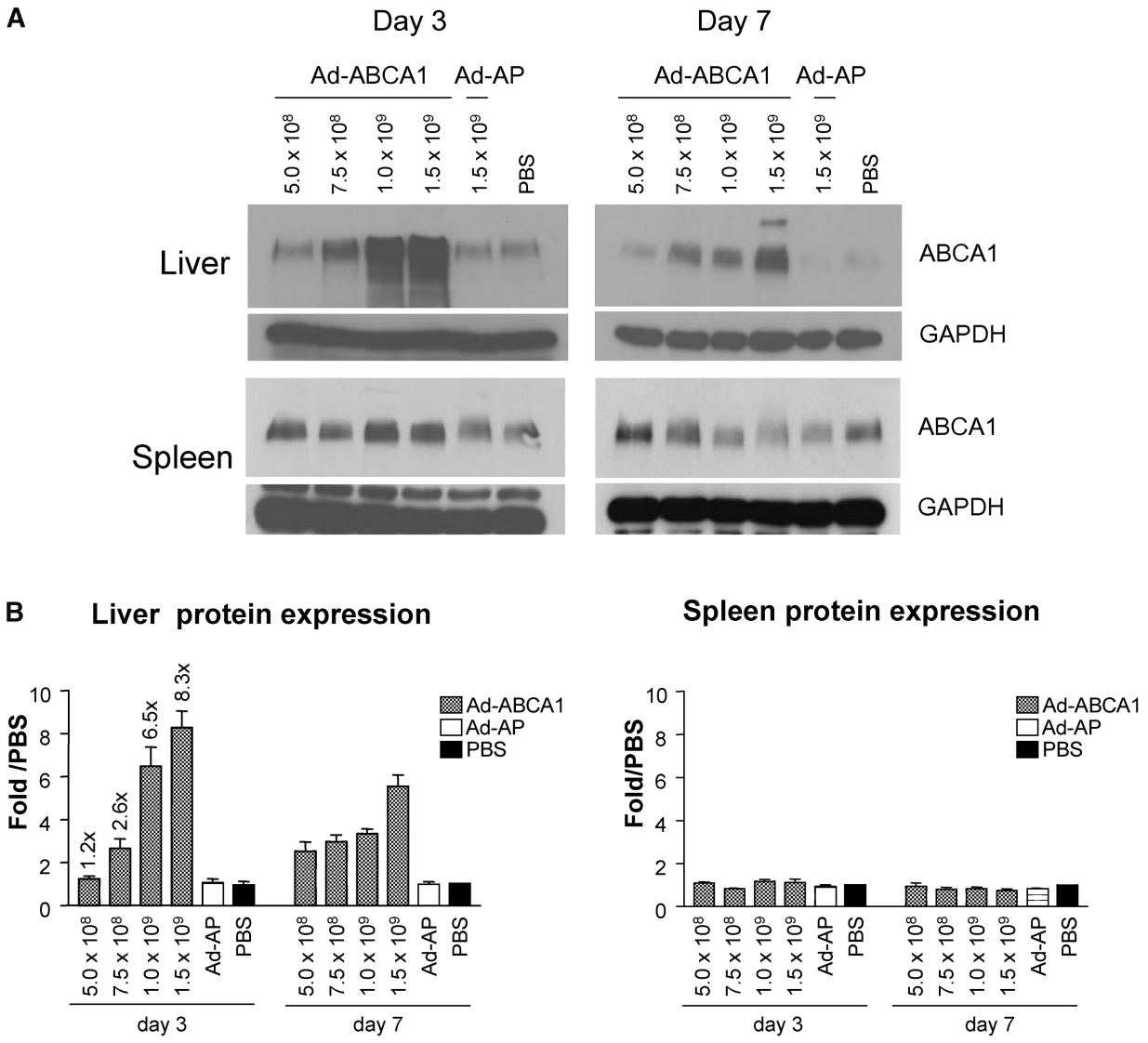

Fig. 2. Dose-dependent increase in ABCA1 expression specifically in the livers of Ad-ABCA1-treated mice. A: Western blots of liver and spleen following injection of mice with the indicated doses of Ad-ABCA1 (5.0×10^8 to 1.5×10^9 pfu/mouse), 1.5×10^9 pfu/mouse of Ad-alkaline phosphatase (AP), or PBS as a vehicle-only control. Total protein lysates prepared from tissues harvested 3 or 7 days postinjection were immunodetected with antibodies specific for ABCA1 and GAPDH to serve as a loading control. B: Quantitation of ABCA1 expression. ABCA1 abundance was normalized to GAPDH levels using densitometry. Pooled data (means and standard deviations) were generated from at least two independent gels for each of the 3–4 mice per group, and expressed relative to endogenous ABCA1 levels in PBS-treated mice.

TABLE 1. Lipid values in Ad-ABCA1-treated mice

Virus	Dose pfu/ Mouse	Day	TC			TG			PL			HDL Cholesterol			non-HDL Cholesterol			apoB		
			Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
											mg/dl									
Ad-ABCA1	5×10^8	3	134.37	3.95	<0.01	122.13	15.25	>0.05	258.30	6.35	>0.05	101.83	7.76	<0.05	32.57	8.95	>0.05	31.04	2.50	>0.05
		7	108.25	4.92	<0.05	136.78	15.05	>0.05	239.25	17.93	>0.05	76.70	5.42	>0.05	31.53	5.09	<0.01	39.72	6.48	<0.001
Ad-ABCA1	7.5×10^8	3	121.95	6.88	>0.05	142.70	24.71	>0.05	240.90	25.47	>0.05	91.22	12.64	>0.05	30.73	7.40	>0.05	39.00	5.91	>0.05
		7	143.38	9.26	<0.001	162.10	11.42	>0.05	304.43	10.88	<0.001	97.45	3.47	<0.05	45.95	7.93	<0.001	44.48	1.36	<0.001
Ad-ABCA1	1×10^9	3	148.90	14.28	<0.001	150.87	45.20	>0.05	284.63	7.54	<0.05	98.17	2.73	>0.05	50.00	8.23	<0.01	39.36	5.69	>0.05
		7	131.08	16.80	<0.001	143.78	19.72	>0.05	273.50	32.62	<0.01	85.65	11.15	>0.05	45.45	10.45	<0.001	41.28	4.46	<0.001
Ad-ABCA1	1.5×10^9	3	183.03	10.73	<0.001	190.03	18.11	>0.05	347.78	24.21	<0.001	111.25	7.32	<0.001	71.75	12.83	<0.001	57.32	3.27	<0.001
		7	155.18	5.12	<0.001	155.85	3.85	>0.05	310.55	7.81	<0.001	94.98	7.02	>0.05	60.18	4.60	<0.001	52.32	4.95	<0.001
Ad-AP	1.5×10^9	3	104.35	4.09	>0.05	139.68	12.33	>0.05	234.50	7.77	>0.05	82.65	5.55	>0.05	21.70	6.81	>0.05	36.24	3.43	>0.05
		7	96.43	21.00	>0.05	112.27	11.04	>0.05	228.77	65.77	>0.05	67.93	9.00	>0.05	28.50	14.80	<0.05	23.57	7.26	>0.05
PBS		3	100.03	15.92		154.35	12.77		220.55	30.35		79.83	16.58		20.20	10.17		38.20	6.94	
		7	82.83	8.35		116.63	19.54		189.50	23.69		75.65	5.79		7.13	3.55		18.72	3.29	

ABCA1, ATP binding cassette transporter A1; Ad, adenovirus; AP, alkaline phosphatase; apoB, apolipoprotein B; TC, total cholesterol; TG, triglyceride; PL, phospholipid. N = 4, *P* relative to PBS-treated mice.

Ad-ABCA1 (5.0×10^8 pfu), which resulted in a 1.2-fold increase in ABCA1 expression (Fig. 2), had a significant 27% increase in plasma HDL cholesterol levels (Table 1, $P < 0.05$). Intermediate doses of exogenous Ad-ABCA1 (7.5×10^8 and 1.0×10^9 pfu) did not result in further significant elevations of plasma HDL cholesterol, despite a 2.6-fold or 6.5-fold increase in hepatic ABCA1 levels, respectively (Fig. 2). HDL cholesterol levels were, however, significantly increased by 39% in mice treated with the highest dose of Ad-ABCA1 (1.5×10^9 pfu) that resulted in an 8.3-fold overexpression in hepatic ABCA1 levels (Table 1, $P < 0.001$). These results confirm that increased hepatic ABCA1 raises HDL cholesterol, in agreement with our previously published findings in chow-fed ABCA1 BAC transgenic mice (19).

However, these results also give the first indication that the normal regulation of ABCA1 is likely to be important for selectively and maximally increasing plasma HDL cholesterol. Compared with the Ad-ABCA1-treated mice, the greatest increase in plasma HDL cholesterol levels was observed in BAC transgenic animals in which a 1.6-fold increase in appropriately expressed ABCA1 protein in the liver was sufficient to increase plasma HDL cholesterol levels by 65% (19). We note that, because the BAC transgenic mice express the ABCA1 transgene using endogenous regulatory sequences contained within the BAC, we cannot exclude the possibility that increased ABCA1 in other tissues may also contribute to the increase in plasma HDL cholesterol levels. For example, intestinal ABCA1 may participate in the regulation of lipoprotein homeostasis, and increased intestinal ABCA1 expression in BAC transgenic mice may also contribute to the increase in HDL cholesterol levels observed in these animals. However, it is likely that ABCA1 in the liver plays a major role in elevation of HDL cholesterol levels in the BAC transgenic mice due not only to tissue mass, but also to the high level of ABCA1 expression compared with other tissues (15).

Excessive hepatic ABCA1 via adenoviral delivery results in a nonselective increase in other plasma lipoprotein levels

In addition to increased HDL cholesterol levels, we observed increased TC, TG, PL, non-HDL cholesterol, and apoB levels in Ad-ABCA1-injected mice compared with controls (Table 1). In mice receiving the highest dose of Ad-ABCA1 (1.5×10^9 pfu), the greatest alterations in plasma lipids were for non-HDL cholesterol levels, which were nearly triple those of control mice at Day 3 ($P < 0.001$), and at least double that of control mice at Day 7 (Table 1, $P < 0.001$). Additionally, we observed a dose-dependent increase in apoB concentration that resulted in a nearly 2-fold increase in apoB levels on Days 3 and 7 in mice treated with 1.5×10^9 pfu of Ad-ABCA1 (Table 1, $P < 0.001$), whereas there were no significant changes in the levels of apoC-III, apoE, and apoA-II (data not shown). Finally, FPLC analysis of cholesterol distribution revealed that the greatest changes in cholesterol due to Ad-ABCA1 treatment were in the LDL range of fractions (**Fig. 3**). Together, these results suggest that the 8.3-fold overexpression of exogenously regulated ABCA1 in mice receiving 1.5×10^9 pfu of Ad-ABCA1 resulted in increased cholesterol in apoB-containing lipoproteins.

There were no marked overall differences in plasma lipids in PBS- or Ad-AP-injected mice, suggesting that the Ad infection per se did not alter plasma lipids. Histological examination of liver sections prepared from Ad-ABCA1- or Ad-AP-treated mice revealed no significant differences at equivalent doses (data not shown). Finally, we observed no change in bile and fecal sterol cholesterol levels in Ad-ABCA1-treated mice compared with Ad-AP or PBS controls (data not shown). These observations suggest that the altered lipid profiles in mice that received high-dose Ad-ABCA1 are unlikely to have arisen from nonspecific changes, including liver damage upon adenoviral infection. Rather, these results suggest that the high levels of ABCA1 overexpression, together with the dissociation of ABCA1 from its normal regulatory circuits, are responsible for the general perturbation in plasma lipids.

A selective increase in HDL cholesterol is best achieved by appropriate regulation of hepatic ABCA1

It is not possible to directly compare the changes in plasma lipids in mice treated with high doses of Ad-ABCA1 ($1.0–1.5 \times 10^9$ pfu) to those observed in the BAC transgenic mice,

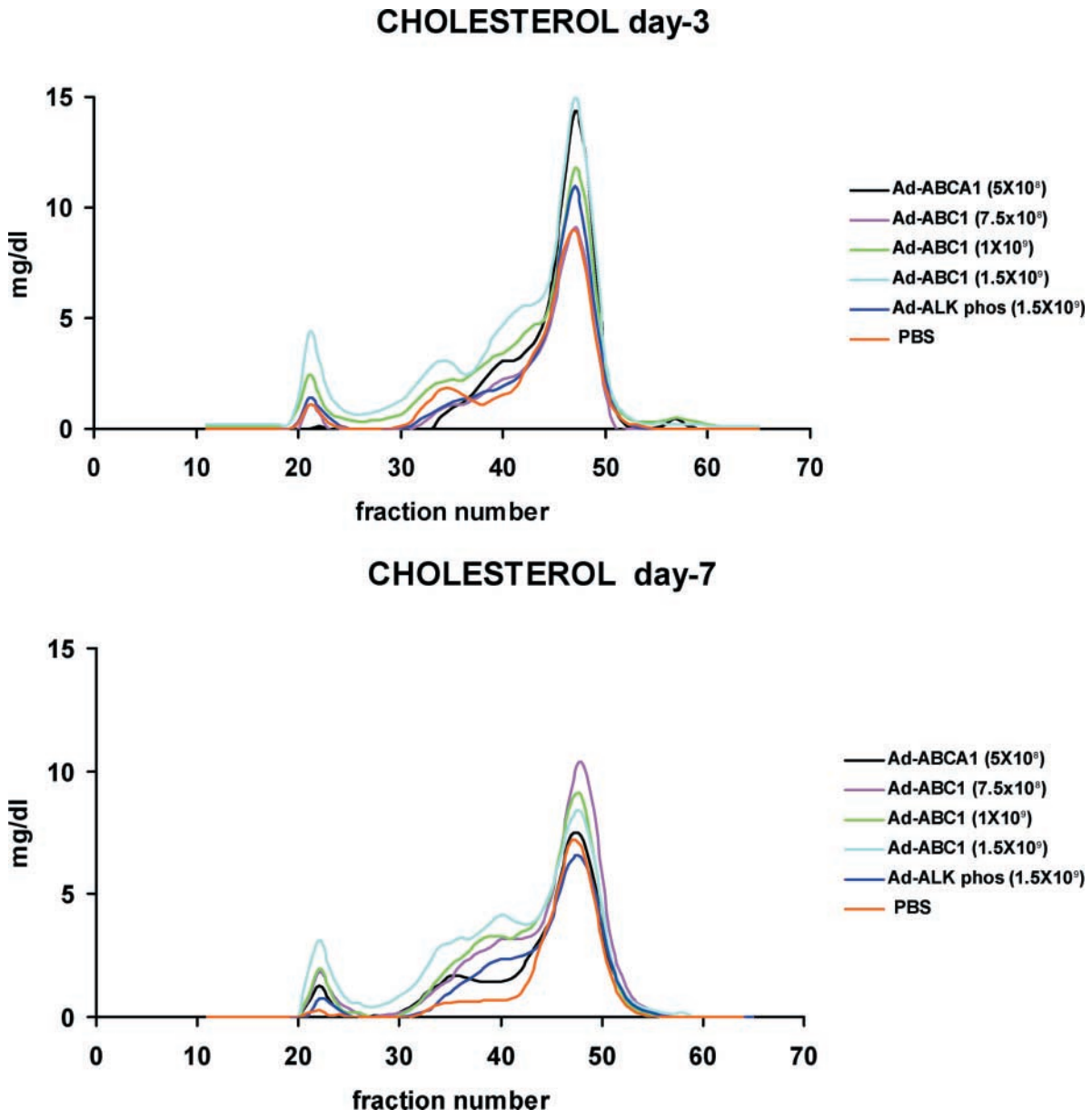

Fig. 3. Cholesterol content of lipoproteins fractionated by FPLC. Plasma samples were collected 3 or 7 days after injection with the indicated dose (pfu/mouse) of Ad-ABCA1, Ad-AP, or PBS, as indicated. Fraction numbers are indicated at the bottom.

because ABCA1 expression is increased 6.5- to 8.3-fold in high-dose Ad-ABCA1-treated mice compared with 1.6-fold in BAC transgenic mice (Fig. 2). However, mice treated with low doses of Ad-ABCA1 (5.0 to 7.5 $\times$ 10^8 pfu) have a 1.2- to 2.6-fold increase in ABCA1 expression (Fig. 2), making them roughly comparable to the BAC transgenic mice in terms of hepatic ABCA1 levels (**Fig. 4**). This observation gave us the opportunity to determine the relative importance of appropriate regulation of ABCA1 in the liver compared with the simple abundance of hepatic ABCA1 in mediating a selective increase in plasma HDL cholesterol levels. We therefore determined the relative changes in plasma lipids observed in BAC transgenic mice compared with nontransgenic controls, and compared these to relative changes in Day 3 plasma lipids observed in Ad-ABCA1-treated or Ad-AP-treated mice, which were normalized to PBS-treated controls (**Fig. 5**).

This analysis revealed that, at roughly comparable expression levels in the liver, TC levels in the BAC transgenic mice tended to be higher than in Ad-ABCA1-treated mice, which was fully accounted for by a selective increase in HDL cholesterol levels (Fig. 5, **Table 2**). For example, consider a comparison between chow-fed BAC transgenic mice that have a 1.6-fold increase of ABCA1 and mice treated with 7.5 $\times$ 10^8 pfu of Ad-ABCA1 that exhibit a 2.6-fold increase in ABCA1. The BAC transgenic mice have a 42% increase in TC ($P < 0.0003$) and a 65% increase in HDL cholesterol levels ($P < 0.005$) compared with wild-type animals (19), whereas the Ad-ABCA1-treated mice have a 22% increase in TC ($P < 0.01$) and a 14% increase in HDL cholesterol levels ($P < 0.05$) compared with controls (Table 1). These results suggest that, despite higher hepatic ABCA1 levels in the Ad-ABCA1-treated mice com-

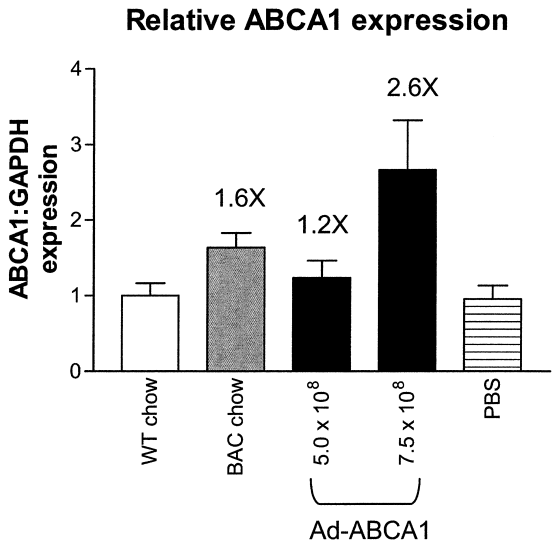

Fig. 4. Comparison of ABCA1 expression levels in the livers of chow-fed bacterial artificial chromosome (BAC) transgenic or Ad-ABCA1-treated mice. ABCA1 levels in total protein lysates prepared from livers of BAC transgenic, Ad-ABCA1-treated (5.0×10^8 or 7.5×10^8 pfu/mouse as indicated), and PBS-treated mice were determined by Western blot analysis from gels from at least three animals per group. All of the mice were maintained on a regular chow diet. ABCA1 levels were quantitated by densitometry and normalized to GAPDH levels in the same lane to control for equal protein loading. The 1.6-fold increase in ABCA1 expression in the BAC transgenic mice was determined relative to wild-type control mice, in which ABCA1 levels were set to 1.0. Similarly, the 1.2-fold and 2.6-fold increases in ABCA1 expression in the Ad-ABCA1-treated mice were determined relative to PBS-treated control mice in which ABCA1 levels were set to 1.0. Bars represent the mean and standard deviations of measurements from at least three mice.

pared with the BAC transgenic mice, adenoviral delivery of ABCA1 was less effective than the endogenously regulated transgene in mediating a selective increase in plasma HDL cholesterol levels in vivo. Moreover, HDL cholesterol levels were consistently higher in the BAC transgenic mice than in Ad-ABCA1-treated mice at any dose tested, further supporting the hypothesis that ABCA1 expressed under the control of endogenous regulatory signals is more likely to selectively increase HDL cholesterol than ABCA1 expressed from an exogenous promoter. The Ad-ABCA1-treated mice are obviously not completely comparable to the BAC transgenic mice, even at comparable expression levels in the liver, because transgenic ABCA1 is also overexpressed in nonhepatic tissues in the BAC transgenic animals. Therefore, we cannot exclude the possibility that appropriately regulated ABCA1 expression in hepatic as well as extrahepatic tissues may play a role in selectively increasing plasma HDL cholesterol levels in the BAC transgenic mice. Conditional transgenic approaches will be required to definitively elucidate the relative importance of hepatic compared with extrahepatic ABCA1 in controlling HDL cholesterol levels.

In addition to a less-marked impact on plasma HDL cholesterol levels, Ad-ABCA1 treatment resulted in changes in other lipids that were not observed in the BAC transgenic mice. For example, chow-fed BAC transgenic mice have no significant changes in non-HDL cholesterol levels compared with wild-type controls (Fig. 5, $P > 0.05$). In comparison, non-HDL cholesterol levels were significantly elevated in mice that received 1.0 to 1.5×10^9 pfu of Ad-ABCA1 (Fig. 5, Table 1). Although non-HDL cholesterol levels were not significantly elevated 3 days after treating mice with the lowest dose of Ad-ABCA1 (5.0×10^8 pfu), there was a significant increase in non-HDL cholesterol in these mice by Day 7 compared with either vehicle or Ad-AP-treated controls (Fig. 5, $P < 0.002$). These results again suggest that even at relatively low expression levels, inappropriate regulation of ABCA1 can lead to changes in plasma lipids other than HDL cholesterol.

DISCUSSION

The discovery of ABCA1 as a critical protein in the generation of HDL led to the hypothesis that an increase in ABCA1 will elevate plasma HDL cholesterol levels and decrease atherosclerosis (1, 9, 14). This hypothesis has been confirmed in the BAC transgenic animal model, where human ABCA1 is expressed from its endogenous regulatory signals on a bacterial artificial chromosome. In these mice, ABCA1 overexpression in chow-fed animals is increased 1.6-fold over endogenous ABCA1, which results in a 65% increase in plasma HDL cholesterol (19). When crossed onto the apoE-knockout model of atherosclerosis, appropriately regulated ABCA1 was found to be dramatically protective against the formation of atherosclerotic plaques (20). In contrast, an ABCA1 cDNA transgenic model showed mixed results with respect to protection from atherosclerosis. In the ABCA1 cDNA transgenic mice, exogenous ABCA1 expression is driven by the apoE promoter that directs ABCA1 overexpression to the liver and macrophages (27). Two lines of mice were generated, with ~30 or 40 copies of the human ABCA1 transgene, which results in a similar 2-fold elevation of plasma HDL cholesterol on a chow diet (27). When these mice were fed a high-fat diet, increased ABCA1 was found to strengthen the atheroprotective lipid profile and reduce atherosclerosis (28). However, increased atherosclerosis was observed when these mice were crossed onto an apoE-deficient background (28). These different results underscore the importance of investigating relationships among ABCA1 regulation, abundance, and expression in selected tissues to plasma HDL cholesterol levels and protection from atherosclerosis in vivo.

To specifically address how hepatic ABCA1 expression contributes to plasma lipids, we used an Ad to selectively overexpress human ABCA1 in the liver and determined the dose-dependent effect of exogenous ABCA1 expression on plasma lipid and lipoprotein profiles. The adenoviral construct used in this study was first validated in vitro by demonstrating efficient apoA-I-dependent cholesterol and PL efflux in both HeLa and HepG2 cells.

In vivo adenoviral delivery of ABCA1 resulted in an in-

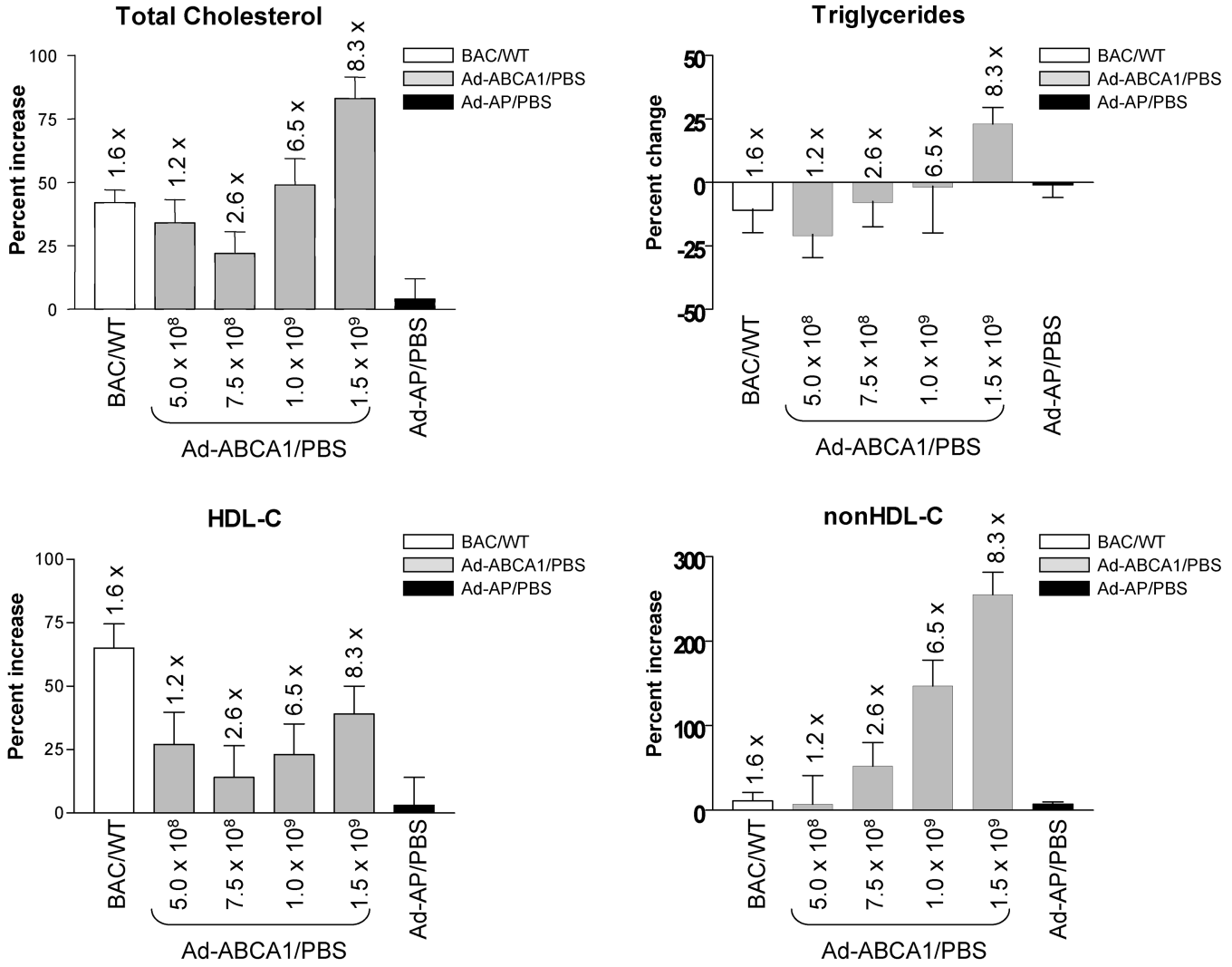

Fig. 5. Relative plasma lipid changes in BAC compared with Ad-ABCA1-treated mice. The percent change in total cholesterol, triglycerides, HDL cholesterol, and non-HDL cholesterol levels were compared among chow-fed BAC transgenic mice, Ad-ABCA1-treated mice, and Ad-AP treated mice. Plasma lipids in BAC transgenic mice were determined relative to nontransgenic controls and expressed as percent change from the baseline observed in the wild-type control animals. Plasma lipids in Ad-ABCA1- or Ad-AP-treated mice were determined relative to those observed mice injected with PBS only and expressed as percent change from the baseline in PBS-treated mice. Viral dose in pfu/mouse is given below the bars, and the fold overexpression of ABCA1 is given above the bars. Data represent the relative means and standard deviations from at least three mice per group.

crease in plasma HDL cholesterol levels as expected, demonstrating that hepatic ABCA1 can participate in HDL cholesterol homeostasis. However, at comparable expression levels, HDL cholesterol was increased by 14–27% in Ad-ABCA1-treated mice compared with a 65% increase in BAC transgenic animals, suggesting that transgenic expression of exogenous ABCA1 from the BAC is more effective in raising plasma HDL cholesterol levels than delivering exogenous ABCA1 with an Ad. In addition, we also observed a striking effect of exogenous ABCA1 on the entire lipid profile, which was most pronounced at high doses of Ad-ABCA1 treatment that resulted in a 8.3-fold overexpression of hepatic ABCA1. For example, the levels of TC, TGs, PLs, non-HDL cholesterol, and apoB were all increased in Ad-ABCA1-treated mice in a dose-dependent manner, and the magnitude of these changes often exceeded that of the increase in HDL cholesterol levels. Interestingly, a similar dose-dependent increase in apoB and non-HDL cholesterol levels was also observed in chow-fed apoE-ABCA1 cDNA transgenic mice, although these increases did not reach significance and it is not possible to distinguish whether these effects are due to nonphysiologic regulation of ABCA1 or high-level ABCA1 expression driven by the apoE promoter (27). Nevertheless, these observations suggest that hepatic ABCA1 can greatly influence overall plasma lipids.

One interpretation of these findings is that the abundance of hepatic ABCA1 protein expression critically affects lipid homeostasis. We observed no impact on non-HDL cholesterol levels in either the BAC transgenic mice or in mice treated with the lowest dose of Ad-ABCA1, which resulted in relatively modest levels of ABCA1 over-

TABLE 2. Percent changes in plasma lipids in bacterial artificial chromosome transgenic compared with Ad-ABCA1-treated mice

Mouse	Virus Dose pfu/Mouse	Fold Increase ABCA1	Percent Change			
			TC	TG	HDL Cholesterol	non-HDL Cholesterol
BAC/WT	na	1.6×	42 ± 10	−11 ± 33	65 ± 19	11 ± 20
Ad-ABCA1/PBS	5.0×10^8	1.2×	34 ± 16	−21 ± 15	27 ± 22	7 ± 59
Ad-ABCA1/PBS	7.5×10^8	2.6×	22 ± 17	−8 ± 19	14 ± 25	52 ± 56
Ad-ABCA1/PBS	1.0×10^9	6.5×	49 ± 18	−2 ± 31	23 ± 21	147 ± 53
Ad-ABCA1/PBS	1.5×10^9	8.3×	83 ± 17	23 ± 13	39 ± 22	255 ± 53
Ad-AP/PBS	1.5×10^9	1.0×	4 ± 16	−1 ± 10	3 ± 22	7 ± 6

BAC, bacterial artificial chromosome.

expression in the liver (1.6-fold and 1.2-fold, respectively). However, increasing the Ad-ABCA1 dose resulted in a clear and dose-dependent increase in non-HDL cholesterol levels. This observation suggests that, although a modest increase in hepatic ABCA1 may preferentially increase HDL cholesterol, excessive ABCA1 may perturb overall lipid homeostasis, perhaps by permitting the transfer of excess free cholesterol to lipoproteins of different subclasses. This effect may only be observed when ABCA1 is highly overexpressed in the absence of appropriate physiological regulatory signals.

A second interpretation is that appropriate regulation of ABCA1 may be critical for selectively increasing HDL cholesterol. In this interpretation, lipid homeostasis is regulated through exquisite control of multiple hepatic genes, including ABCA1. Because the Ad-ABCA1 vector used in this study contained only the full-length human ABCA1 cDNA, it is likely that this construct lacked elements that ultimately regulate the appropriate targeting of ABCA1 to the basolateral membrane (29, 30) or to direct efficient posttranslational modification of ABCA1 (24). Appropriate subcellular localization and/or modification of ABCA1 may play important roles in appropriate lipidation of apoA-I.

It is also possible that coordinated ABCA1 expression in multiple tissues may be required to observe a selective increase in plasma HDL cholesterol levels. In the BAC transgenic mice, the presence of endogenous regulatory signals ensures that transgenic ABCA1 is overexpressed in all tissues where it is normally expressed, therefore resulting in a coordinated overexpression of ABCA1 throughout the body. It is possible that the combined effects of hepatic and extrahepatic ABCA1 contribute to the efficient increase in HDL cholesterol levels in the BAC transgenic mice compared with Ad-ABCA1-treated mice at comparable expression levels. Because macrophage-specific ABCA1 has been shown to contribute minimally to plasma HDL cholesterol levels in vivo (18), it is possible that other extrahepatic tissues such as the intestine may impact HDL cholesterol homeostasis in vivo through ABCA1 activity.

While this manuscript was in preparation, Basso et al. reported their findings on adenoviral delivery of ABCA1 to mice (31) (**Table 3**). Although both groups used the same mouse strain (C57Bl/6), the constructs were different. Basso et al. used a murine ABCA1 cDNA fused to GFP, whereas we used an unmodified human ABCA1 cDNA. The dose used by Basso et al. ($1–5 \times 10^8$ pfu/mouse), which resulted in an increase in ABCA1 protein by 1.6- to 1.7-fold within 24 h (31), is most comparable to the lowest dose used in our study (5×10^8 pfu/mouse), which resulted in a 1.2-fold increase in ABCA1 protein by 3 days.

Basso et al. observed maximal changes in plasma lipids 2 days after treatment, with the major effects being a 2- to 3-fold increase in HDL cholesterol, TC, PL, FC, and CE levels, and a significant increase in α-HDL particles (31). In their study, there was little apparent effect on non-HDL cholesterol levels, in contrast to their previous observations in the apoE-ABCA1 cDNA transgenic mice (27, 31). Additionally, Basso et al. observed significant increases in HMG-CoA reductase, LDL receptor, and LDL receptor-related protein expression levels that were detectable in as

TABLE 3. Comparison of results of adenoviral delivery of ABCA1 studies in vivo

Variable	Basso et al.	Wellington et al.
Mouse strain	C57Bl/6	C57Bl/6
Age	2–4 months	6–8 months
Adenoviral promoter	TRE/minCMV	CMV
Species of ABCA1	murine	human
Construct	ABCA1-GFP	ABCA1
Dose	$1–5 \times 10^8$ pfu/mouse	$5 \times 10^8–1.5 \times 10^9$ pfu/mouse
Time of analysis	6 h to 4 days post treatment	3 and 7 days post treatment
ABCA1 expression	1.6-fold	1.2- to 8.3-fold, increasing by dose
TC	2- to 3-fold	1.3- to 1.8-fold, increasing by dose
HDL cholesterol	2- to 3-fold	1.3- to 1.4-fold, increasing by dose
non-HDL cholesterol	na	1.6- to 3.5-fold, increasing by dose

CMV, cytomegalovirus.

little as 6 h after Ad-ABCA1-GFP treatment (31). We detected maximal changes in plasma lipids at Day 3 after treatment, suggesting that the changes in plasma lipid levels appeared to follow similar dynamics in the two studies.

Importantly, comparison of the relative changes in plasma lipids in the BAC transgenic mice and Ad-ABCA1-treated mice at roughly comparable expression levels showed that HDL cholesterol levels were selectively increased more efficiently in the BAC transgenic model. This observation suggests that regulation of ABCA1, in the liver and in other tissues, may be critical for effectively raising HDL cholesterol levels. ABCA1 may also function coordinately with other genes help to maintain specificity to HDL. In the Ad-ABCA1-treated mice, increased ABCA1 expression is uncoupled from other hepatic genes, as well as other tissues that may also contribute to regulating HDL cholesterol levels.

Endogenous ABCA1 is under exquisite regulation at transcriptional and posttranslational levels, and appropriate levels in multiple tissues, subcellular location within cells of particular tissues, and interaction with other cellular proteins are likely to be critical parameters for the ability of ABCA1 to selectively increase HDL cholesterol levels. Furthermore, ABCA1 activity in multiple tissues may also affect HDL cholesterol homeostasis in vivo. Our results suggest that therapeutic approaches based on increasing ABCA1 expression will likely have the greatest beneficial effect if they target endogenous pathways that maintain appropriate regulation of ABCA1.

The authors are grateful to Alan Attie, Angie Tebon, and Sherrie Tafuri for their many insightful comments during the course of this study. This work is supported by the Canadian Institutes of Health Research (M.R.H. and R.J.P.), the Heart and Stroke Foundation of Canada (M.R.H.), the Canadian Networks of Centres of Excellence (NCE Genetics, M.R.H.), and Xenon Genetics Inc. R.J.P. is a CIHR New Investigator, and M.R.H. is a holder of a Canada Research Chair.

REFERENCES

1. Hayden, M. R., S. M. Clee, A. Brooks-Wilson, J. Genest, Jr., A. Attie, and J. J. P. Kastelein. 2000. Cholesterol efflux regulatory protein, Tangier disease and familial high-density lipoprotein deficiency. *Curr. Opin. Lipidol.* **11:** 117–122.
2. Schmitz, G., and T. Langmann. 2001. Structure, function and regulation of the ABC1 gene product. *Curr. Opin. Lipidol.* **12:** 129–140.
3. Wang, N., D. L. Silver, C. Theile, and A. R. Tall. 2001. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J. Biol. Chem.* **276:** 23742–23747.
4. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
5. Fielding, C. J., and P. E. Fielding. 1997. Intracellular cholesterol transport. *J. Lipid Res.* **38:** 1503–1521.
6. Fielding, C. J., and P. E. Fielding. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* **36:** 211–228.
7. Rothblat, G. H., M. de la Llera-Moya, V. Atger, G. Kellner-Weibel, D. L. Williams, and M. C. Phillips. 1999. Cell cholesterol efflux: integration of old and new observations provides new insights. *J. Lipid Res.* **40:** 781–796.
8. Tall, A. R., N. Wang, and P. Mucksavage. 2001. Is it time to modify the reverse cholesterol transport model? *J. Clin. Invest.* **108:** 1273–1275.
9. Brooks-Wilson, A., M. Marcil, S. M. Clee, L. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J. A. Collins, H. O. F. Molhuizen, O. Loubser, B. F. F. Ouellette, K. Fichter, K. J. D. Ashbourne Excoffon, C. W. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. N. Pimstone, J. J. P. Kastelein, J. Genest, Jr., and M. R. Hayden. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22:** 336–345.
10. Marcil, M., A. Brooks-Wilson, S. M. Clee, K. Roomp, L. Zhang, L. Yu, J. A. Collins. M. van Dam, H. O. F. Molhuizen, O. Loubser, B. F. F. Ouellette, C. W. Sensen, K. Fichter, S. Mott, M. Denis, B. Boucher, S. Pimstone, J. Genest, Jr., J. J. P. Kastelein, and M. R. Hayden. 1999. Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet.* **354:** 1341–1346.
11. Bodzioch, M., E. Orsó, J. Klucken, T. Langmann, A. Böttcher, W. Diederich, W. Drobnik, S. Barlage, C. Büchler, M. Porsch-Özcürümez, W. E. Kaminski, H. W. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K. J. Lackner, and G. Schmitz. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier Disease. *Nat. Genet.* **22:** 347–351.
12. Rust, S., M. Rosier, H. Funke, Z. Amoura, J-C. Piette, J-F. Deleuze, H. B. Brewer, Jr., N. Duverger, P. Denèfle, and G. Assmann. 1999. Tangier Disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* **22:** 352–355.
13. Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan, and J. F. Oram. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
14. Clee, S. M., J. J. P. Kastelein, M. van Dam, M. Marcil, K. Roomp, K. Y. Zwarts, J. A. Collins, R. Roelants, N. Tamasawa, T. Stulc, T. Suda, R. Ceska, B. Boucher, C. Rondeau, C. DeSouich, A. Brooks-Wilson, H. O. F. Molhuizen, J. Frohlich, J. Genest, Jr., and M. R. Hayden. 2000. HDL cholesterol levels and coronary artery disease in heterozygotes for ABCA1 mutations are predicted by cholesterol efflux levels and influenced by age. *J. Clin. Invest.* **106:** 1263–1270.
15. Wellington, C. L., E. K. Walker, A. Suarez, A. Kwok, N. Bissada, R. Singaraja, Y-Z. Yang, L. H. Zhang, E. James, J. E. Wilson, O. Francone, B. M. McManus, and M. R. Hayden. 2002. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. *Lab. Invest.* **82:** 273–283.
16. Lawn, R. M., D. P. Wade, T. L. Couse, and J. N. Wilcox. 2001. Localization of human ATP-binding cassette transporter 1 (ABC1) in normal and atherosclerotic tissues. *Arter. Thromb Vasc. Biol.* **21:** 378–385.
17. Attie, A. D., Y. Hamon, A. Brooks-Wilson, M. P. Gray-Keller, M. L. MacDonald, V. Rigot, A. Tebon, L. H. Zhang, J. D. Mulligan, R. R. Singaraja, J. J. Bitgood, M. E. Cook, J. J. Kastelein, G. Chimini, and M. R. Hayden. 2002. Identification and functional analysis of a naturally occuring E89K mutation in the ABCA1 gene of the WHAM chicken. *J. Lipid Res.* **43:** 1610–1617.
18. Haghpassand, M., P-A. K. Bourassa, O. L. Francone, and R. J. Aiello. 2001. Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J. Clin. Invest.* **108:** 1315–1320.
19. Singaraja, R. R., V. Bocher, E. R. James, S. M. Clee, L-H. Zhang, B. R. Leavitt, B. Tan, A. Brooks-Wilson, A. Kwok, N. Bissada, Y-Z. Yang, G. Liu, S. R. Tafuri, C. Fievet, C. L. Wellington, B. Staels, and M. R. Hayden. 2001. Human ABCA1 BAC transgenic mice show increased HDL-C and ApoA1-dependent efflux stimulated by an internal promoter containing LXREs in intron 1. *J. Biol. Chem.* **276:** 33969–33979.
20. Singaraja, R., C. Fievet, G. Castro, E. R. Jamers, N. Hennuyer, S. M. Clee, N. Bissada, J. C. Choy, J-C. Fruchart, B. M. McManus, and M. R. Hayden. 2002. Increased ABCA1 activity protects against atherosclerosis. *J. Clin. Invest.* **110:** 35–42.
21. Liu, G., K. J. D. Ashbourne-Excoffon, J. E. Wilson, B. M. McManus, Q. R. Rogers, L. Miao, J. J. Kastelein, M. E. S. Lewis, and M. R. Hayden. 2000. Phenotypic correction of feline lipoprotein lipase deficiency by adenoviral gene transfer. *Human Gene Therapy.* **11:** 21–32.
22. Nishida, Y., K. Hirano, K. Tsukamoto, M. Nagano, C. Ikegami, K. Roomp, M. Ishihara, N. Sakane, Z. Zhang, K. Tsujii, A. Matsuyama, T. Ohama, F. Matsuura, M. Ishigami, N. Sakai, H. Hiraoka, H. Hattori, C. Wellington, Y. Yoshida, S. Misugi, M. R. Hayden, T. Egashira, S. Yamashita, and Y. Matsuzawa. 2002. Expression and functional analyses of novel mutations of ATP-binding cassette transporter-1 in Japanese patients with high-density lipoprotein deficiency. *Biochem. Biophys. Res. Commun.* **290:** 713–721.

23. Wellington, C. L., Y-Z. Yang, S. Zhou, S. M. Clee, B. Tan, K. Hirano, K. Zwarts, A. Kwok, A. Gelfer, M. Marcil, S. Newman, K. Roomp, R. Singaraja, J. Collins, L. H. Zhang, A. K. Groen, K. Hovingh, A. Brownlie, S. Tafuri, J. Genest, Jr., J. J. Kastelein, and M. R. Hayden. 2002. Truncation mutations in ABCA1 suppress normal upregulation of full-length ABCA1 by 9-cis-retinoic acid and 22-R-hydroxycholesterol. *J. Lipid Res.* **43:** 1939–1949.
24. See, R. H., R. A. Caday-Malcolm, R. R. Singaraja, S. Zhou, A. Silverston, M. T. Huber, J. Moran, E. R. James, R. Janoo, J. M. Savill, V. Rigot, L-H. Zhang, M. Wang, G. Chimini, C. L. Wellington, S. R. Tafuri, and M. R. Hayden. 2002. Protein kinase A site-specific phosphorylation regulates ATP-binding cassette A1 (ABCA1)-mediated phospholipid efflux. *J. Biol. Chem.* **277:** 41835–41842.
25. Plösch, T., T. Kok, V. W. Bloks, M. J. Smit, R. Havinga, G. Chimini, A. K. Groen, and F. Kuipers. 2002. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J. Biol. Chem.* **277:** 33870–33877.
26. Li, Q., M. A. Kay, M. Finegold, L. D. Stratford-Perricaudet, and S. L. Woo. 1993. Assessment of recombinant Ad vectors for hepatic gene therapy. *Hum. Gene Ther.* **4:** 403–409.
27. Vaisman, B. L., G. Lambert, M. Amar, C. Joyce, T. Ito, R. D. Shamburek, W. J. Cain, J. Fruchart-Najib, E. D. Neufeld, A. T. Remaley, H. B. Brewer, Jr., and S. Santamarina-Fojo. 2001. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J. Clin. Invest.* **108:** 303–309.
28. Joyce, C. W., M. J. A. Amar, G. Lambert, B. L. Vaisman, B. Paigen, J. Najib-Fruchart, R. F. Hoyt, Jr., E. D. Neufeld, A. T. Remaley, D. S. Fredrickson, and H. B. Brewer, Jr. 2002. The ATP binding cassette transporter A1 (ABCA1) modulates the development of aortic atherosclerosis in C57BL/6 and apoE-knockout mice. *Proc. Natl. Acad. Sci. USA.* **99:** 407–412.
29. Neufeld, E. D., S. J. Demosky, J. A. Stonik, C. Combs, A. T. Remaley, N. Duverger, S. Santamarina-Fojo, and H. B. Brewer, Jr. 2002. The ABCA1 transporter functions on the basolateral surface of hepatocytes. *Biochem. Biophys. Res. Commun.* **297:** 974–979.
30. Mulligan, J. D., M. T. Flowers, A. Tebon, J. J. Bitgood, C. L. Wellington, M. R. Hayden, and A. D. Attie. 2003. ABCA1 is essential for efficient basolateral cholesterol efflux during the absorption of dietary cholesterol in chickens. *J. Biol. Chem.* **278:** 13356–13366.
31. Basso, F., L. Freeman, C. L. Knapper, A. Remaley, J. Stonik, E. B. Neufeld, T. Tansey, M. J. A. Amar, J. Fruchart-Najib, N. Duverger, S. Santamarina-Fojo, and H. B. Brewer, Jr. 2003. Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL-cholesterol concentrations. *J. Lipid Res.* **44:** 296–302.