ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation

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Summary

Here we demonstrate that the ABC transporter ABCG1 plays a critical role in lipid homeostasis by controlling both tissue lipid levels and the efflux of cellular cholesterol to HDL. Targeted disruption of *Abcg1* in mice has no effect on plasma lipids but results in massive accumulation of both neutral lipids and phospholipids in hepatocytes and in macrophages within multiple tissues following administration of a high-fat and -cholesterol diet. In contrast, overexpression of human ABCG1 protects murine tissues from dietary fat-induced lipid accumulation. Finally, we show that cholesterol efflux to HDL specifically requires ABCG1, whereas efflux to apoA1 requires ABCA1. These studies identify *Abcg1* as a key gene involved in both cholesterol efflux to HDL and in tissue lipid homeostasis.

Introduction

The reverse cholesterol transport pathway is critical for the transfer of excess cholesterol from peripheral tissues back to the liver for subsequent catabolism and excretion (Fielding and Fielding, 2001). The initial efflux of cellular cholesterol from these tissues is thought to be dependent upon extracellular lipid acceptors that include HDL and/or lipid-poor apoproteins (Fielding and Fielding, 2001). Although plasma HDL levels are inversely related to the risk of atherosclerosis (Hayden et al., 2000), it has yet to be established that the reverse cholesterol transport pathway plays a critical role in preventing the formation of cholesterol-loaded macrophages in the artery wall.

ABCG1 and ABCA1 are members of the ATP binding cassette (ABC) superfamily of transmembrane transporters that pump specific substrates across membranes (Borst and Elferink, 2002; Dean et al., 2001). Both the *ABCG1* and *ABCA1* genes are expressed in numerous tissues and are highly activated by the nuclear receptor LXR (Costet et al., 2000; Kennedy et al., 2001; Lorkowski et al., 2001; Schwartz et al., 2000; Venkateswaran et al., 2000). Extensive studies have reported that ABCA1 promotes the efflux of both cholesterol- and choline-containing phospholipids to lipid-poor apolipoproteins that include apoAI, apoCII, or apoE (Attie et al., 2001; Bortnick et al., 2000; Oram and Lawn, 2001; Remaley et al., 1995; Schmitz and Langmann, 2001; Yancey et al., 2003).

The physiological importance of ABCA1 has been established from studies on Tangier patients, who have two mutant *ABCA1* alleles (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Lawn et al., 1999; Rust et al., 1999). In addition, studies utilizing *Abca1*-null mice or bone marrow transplantation provided important insights into the role of ABCA1 in effluxing cholesterol to apoA1 (Aiello et al., 2003; Christiansen-Weber et al., 2000; Haghpassand et al., 2001; McNeish et al., 2000). Together these studies demonstrated that (1) normal plasma HDL levels are dependent upon expression of ABCA1 and (2) macrophage ABCA1 has a minor effect on plasma HDL levels but is important as a mediator of lipid efflux to exogenous lipid-poor apoproteins.

In contrast to ABCA1, the function and importance of ABCG1 in lipid homeostasis in vivo is unknown. In vitro studies demonstrated that treatment of peritoneal macrophages with antisense oligonucleotides resulted in decreased expression of a 110 kDa protein and decreased cholesterol efflux to HDL_3 (Klucken et al., 2000). However, the exact cellular and physiological function of ABCG1 remained unclear as the antisense oligonucleotides also resulted in a significant decline in apoE secretion (Schmitz et al., 2001).

Both the human and murine *ABCG1* genes utilize multiple promoters and alternative splicing to produce a diverse array of mRNAs that encode multiple putative protein isoforms that range from 64–79 kDa (Kennedy et al., 2001; Lorkowski et al., 2001; Nakamura et al., 2004). It is not known if these isoforms, which vary only at the amino terminus upstream of the Walker A motif, form distinct dimers and/or have different functions in vivo.

We report here both loss-of-function and gain-of-function studies of *Abcg1* using mice with either targeted disruption of the *Abcg1* locus or expressing the human *ABCG1* BAC transgene. The data indicate that ABCG1 plays a critical role

in controlling tissue lipid levels by mediating the transfer of cellular cholesterol to HDL.

Results

Characterization of *Abcg1*-null/*lacZ* knockin mice

To characterize the physiological function of ABCG1, mice harboring a *lacZ* cassette insertion in exon 3 of the *Abcg1* locus were studied (Figure S1A available with this article online). The original *Abcg1*$^{+/-}$ mice were obtained from Deltagen Inc., San Carlos, California. The β-galactosidase gene, modified to contain a nuclear targeting signal, was under the control of the *Abcg1* promoters, thus allowing facile determination of tissue and cell type expression and regulation by the endogenous promoters. PCR analysis of genomic DNA allowed rapid identification of heterozygous and homozygous mice (Figure S1B). ABCG1 mRNA was undetectable in the lungs of *Abcg1*$^{-/-}$ mice (Figure S1C). However, Northern analysis showed approximately equal ABCG1 mRNA levels in tissues of wild-type and heterozygous mice, suggesting that a single allele can maintain wild-type levels of expression (Figure S1C and data not shown). Analysis of young *Abcg1*$^{-/-}$ mice (<7 weeks old) fed a chow diet failed to detect any significant changes in body weight, blood lipids, blood chemistry, or tissue pathology (data not shown). *Abcg1*$^{+/-}$ and Abcg1$^{-/-}$ mice exhibited normal fertility. In addition, *Abcg1*$^{-/-}$ pups exhibit normal viability and growth. These data contrast with the nonproductive mating and neonatal deaths that have been observed with *Abca1*$^{-/-}$ mice (Christiansen-Weber et al., 2000).

Cellular expression and regulation of *Abcg1*/*lacZ* in *Abcg1*$^{+/-}$ mice

To gain insight into the tissue and cellular expression pattern of ABCG1, we analyzed frozen tissue sections from *Abcg1*$^{+/-}$ mice. Tissue was taken from chow-fed animals and those whose diet had been supplemented with the LXR ligand T0901317. This allowed us to characterize both basal and LXR-activated levels of expression of the gene. LacZ was detected in numerous tissues of chow-fed *Abcg1*$^{+/-}$ mice, with particularly high levels being observed in macrophages in the ileum, liver, and lung (Figures 1A, 1C, and 1E). Scattered lacZ-positive stained cells were also observed in both the follicles and interfollicular regions of the spleens of chow-fed animals (Figure 1G), consistent with the expression of ABCG1 in both macrophages and lymphocytes. Significant lacZ activity was also noted in endothelial cells lining the vasculature of the liver and kidney medulla (Figures 1C, 1D, and 1L). Epithelial cells that comprise the distal collecting tubules of the kidney were also positive (Figure 1I–1L). The data of Figure 1 (panels B, F, H, and J) show that administration of the LXR ligand T0901317 to *Abcg1*$^{+/-}$ mice increased lacZ activity in a number of tissues, but especially the ileum, lung, spleen, and kidney. These analyses identified previously unknown expression patterns for ABCG1 outside of macrophages.

The absence of lacZ activity in hepatocytes of *Abcg1*$^{+/-}$ or *Abcg1*$^{-/-}$ mice fed chow or chow supplemented with T0901317 (Figures 1C and 1D; data not shown for *Abcg1*$^{-/-}$) was unexpected and suggested that lacZ expression in hepatocytes might be below the level of detection. Consequently, we performed in situ hybridization studies utilizing liver sections from wild-type mice fed chow or chow + T0901317 (Figure 2). All

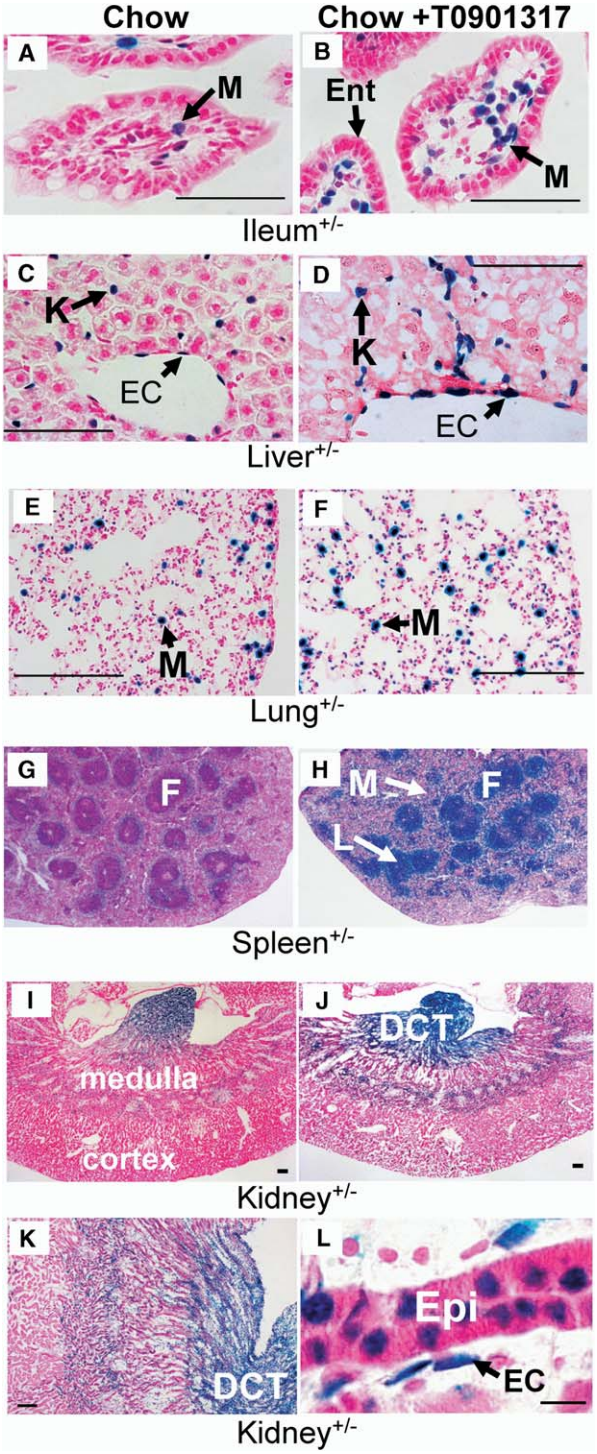

Figure 1. LacZ expression in tissues of *Abcg1*$^{+/-}$ mice

Abcg1$^{+/-}$ mice were fed a normal diet or a diet supplemented with T0901317 for 10 days. Sections of the ileum (**A** and **B**), liver (**C** and **D**), lung (**E** and **F**), spleen (**G** and **H**), and kidney (**I–L**) from *Abcg1*$^{+/-}$ mice were stained for lacZ activity (blue) as described. Macrophages (M), enterocytes (Ent), epithelial cells (Epi), Kupffer cells (K), distal collecting tubules (DCT), and endothelial cells (EC) are indicated. Lymphocytes (L) are present in the spleen follicles (F). Bars: 100 μm (**A–F**, **I–K**) or 20 μm (**L**).

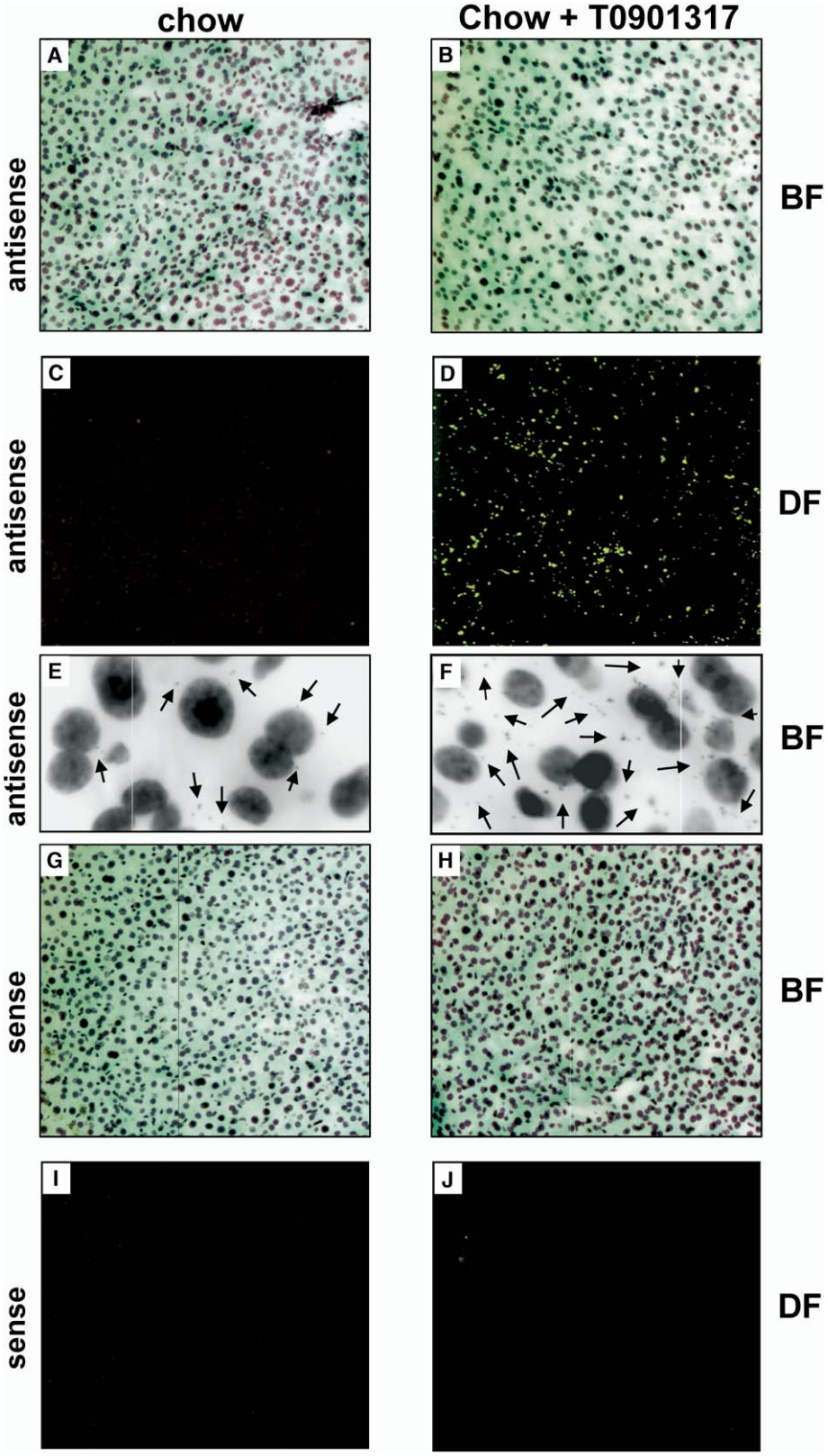

Figure 2. In situ hybridization studies indicate that ABCG1 mRNA is expressed in hepatocytes

Wild-type mice were fed chow (left panels) or chow + T0901317 (50 mg/kg/day) (right panels) for 10 days. Liver sections were incubated with antisense or sense probes, as described in Experimental Procedures. The bright field (BF) and dark field (DF) from identical sections identify the cell nuclei and the signal from the antisense or sense probes, respectively. (**A**)–(**D**) and (**G**)–(**J**) are shown at 125× magnification. Positive signals that identify ABCG1 mRNA in the bright field (**E** and **F**; 500× magnification) are indicated by arrows.

liver sections contained similar concentrations of cells, as judged by nuclear staining and analysis under bright field (Figures 2A, 2B, 2G, and 2H). The data of Figure 2 (panels C and E) show that ABCG1 mRNA is expressed at very low levels throughout the livers of wild-type mice fed a chow diet. As expected, ABCG1 mRNA levels increased significantly following administration of T0901317 to the mice (Figures 2D and 2F). Importantly, most, if not all, hepatic cells expressed ABCG1 mRNA (Figures 2C–2F). No signal was observed when a sense oligonucleotide was utilized (Figures 2I and 2J). Since hepatocytes represent the major cell type in the liver, these data, together with the data of Figure 1, demonstrate that ABCG1 is expressed in Kupffer cells, endothelial cells, and hepatocytes.

Abcg1$^{-/-}$ mice fed a high-fat/high-cholesterol diet fail to maintain normal tissue lipid levels

Given that no striking phenotype was noted in young *Abcg1*-null animals on a standard diet, and ABCG1 is activated by LXR in response to lipid loading in macrophages, we fed mice a diet high in fat and cholesterol. If ABCG1 is involved in the normal efflux of lipids from cells, then lipid accumulation should be observed in *Abcg1*-null animals. To test this hypothesis, age- and sex-matched animals were fed a diet containing 21% fat and 1.25% cholesterol for nine weeks. Despite the fact that there was no change in plasma lipid levels (Figure S2), the *Abcg1*-null mice displayed profound changes in histology of several tissues. For example, visual examination of the lungs of the *Abcg1*-null mice indicated that this organ was abnormal as the lung surface appeared highly irregular and contained numerous large indentations/crevices. In addition many pale foci, indicative of lipid accumulation, were observed (data not shown). Given the gross physical difference between the lungs of the *Abcg1*-null and wild-type animals, we were prompted to examine the pathology of this organ in more detail. In particular, it was of interest to determine if the pale foci were indeed the result of lipid accumulation.

Consequently, frozen lung sections were prepared from mice fed the high-fat/high-cholesterol diet and stained for β-galactosidase activity. As expected, the lungs of wild-type mice showed no evidence of lacZ expression or lipid accumulation (Figures 3Aa and 3Ab). In contrast, not only did all macrophages in the lungs of *Abcg1*-null mice stain positive for lacZ, but it was apparent that a significant number were localized to the subpleural region of the lung (Figure 3Ac). Of greater significance was the fact that this was the same area that showed massive neutral lipid accumulation, as seen by oil red O staining (Figure 3Ad). The subpleural region of the lungs of *Abcg1*$^{-/-}$ mice also contained a plethora of cholesterol clefts, many lacZ-positive multinucleated giant cells, and lymphocytes (Figures 3Ba and 3Bb). LacZ expression was also observed in the epithelial cells that line the bronchioles and endothelial cells that line the blood vessels (Figure 3Bc). Taken together, these results implicate a role for ABCG1 in both macrophages and lymphocytes in response to a high-fat/high-cholesterol diet.

Ultrastructural analysis using electron microscopy revealed the presence of classic macrophage foam cells in the subpleural region of *Abcg1*-null lungs (Figure 3C). In addition to large numbers of lipid droplets, many foam cells contained remarkable numbers of cholesterol clefts (Figure 3C), consistent with a severe defect in the normal processing of this lipid. This is significant because it suggests that ABCG1 is not involved in the uptake of lipid but rather in the subsequent processing and excretion of the lipid from these cells. Finally, a pneumocyte (alveolar) type II cell, which functions to secrete lamellar bodies containing surfactant, was easily discerned in the micrograph (Figure 3C). A few lamellar bodies can be seen within the macrophage, consistent with the normal uptake of these bodies by phagocytic cells (Morgenroth, 1988).

Excessive neutral lipid accumulation in response to the high-fat/high-cholesterol diet was not limited to the lung or to macrophages. Lipid accumulation and oil red O staining was consistently far greater in the livers of *Abcg1*-null as compared to wild-type animals (Figure 3Db versus 3Da). However, unlike the lung where lipid accumulation was restricted to macrophages, the neutral lipids accumulated in both resident macrophages (Kupffer cells) and hepatocytes (Figure 3D). These data suggest that the very low mRNA levels of ABCG1 that are expressed in murine (Figure 2) or rat hepatocytes (Hoekstra et al., 2003) are critical for lipid homeostasis.

The ileum of control and *Abcg1*-null mice fed the high-fat/high-cholesterol diet did not stain with oil red O (Figure S3), suggesting that lipid absorption may be independent of ABCG1 expression that was limited to macrophages within the lamina propria (Figures 1A and 1B).

Examination of kidney tissue also revealed a complete absence of oil red O staining (data not shown). In this regard, it may be significant that ABCG1/lacZ expression in the kidney was restricted to epithelial and endothelial cells and no evidence of lacZ-positive macrophages was observed (Figures 1I–1L). In contrast to *Abca1*-null mice (Christiansen-Weber et al., 2000), the kidneys of *Abcg1*$^{-/-}$ mice showed no evidence of glomerulonephritis or inflammatory cell infiltration.

Characterization of the human *ABCG1*-transgenic mice

Based on the results with *Abcg1*$^{-/-}$ mice we hypothesized that transgenic mice harboring a BAC containing the intact human *ABCG1* gene would be protected from the effects of the high-fat diet. Consequently, we generated C57Bl/6-transgenic mice that contained a single copy of a human BAC (145 kb). The human *ABCG1* gene (100 kb) and 35 kb 5′ and 10 kb 3′ of the gene are contained within this BAC. In order to determine whether the human *ABCG1* gene was expressed and regulated in appropriate tissues, transgenic mice and wild-type littermates were fed chow or chow supplemented with the LXR ligand T0901317. The data of Figure 4 (panels A–E) show that, in response to T0901317, the levels of both the human and murine ABCG1 mRNAs were induced 5- to 14-fold in the lung, liver, kidney, ileum, and spleen. Real-time quantitative PCR utilizing RNA isolated from murine or human macrophages (RAW and THP1, respectively) show that the probes were highly specific (Figure 4F). Importantly, the data of Figures 4A–4E demonstrate that overexpression of the human ABCG1 mRNA did not affect the basal or the T0901317-dependent induction of the murine *Abcg1* gene. Since T0901317 induced the expression of the human *ABCG1* gene, we conclude that the BAC clone contained the appropriate *cis* elements necessary for transcriptional activation of human *ABCG1* in response to LXR-specific ligands.

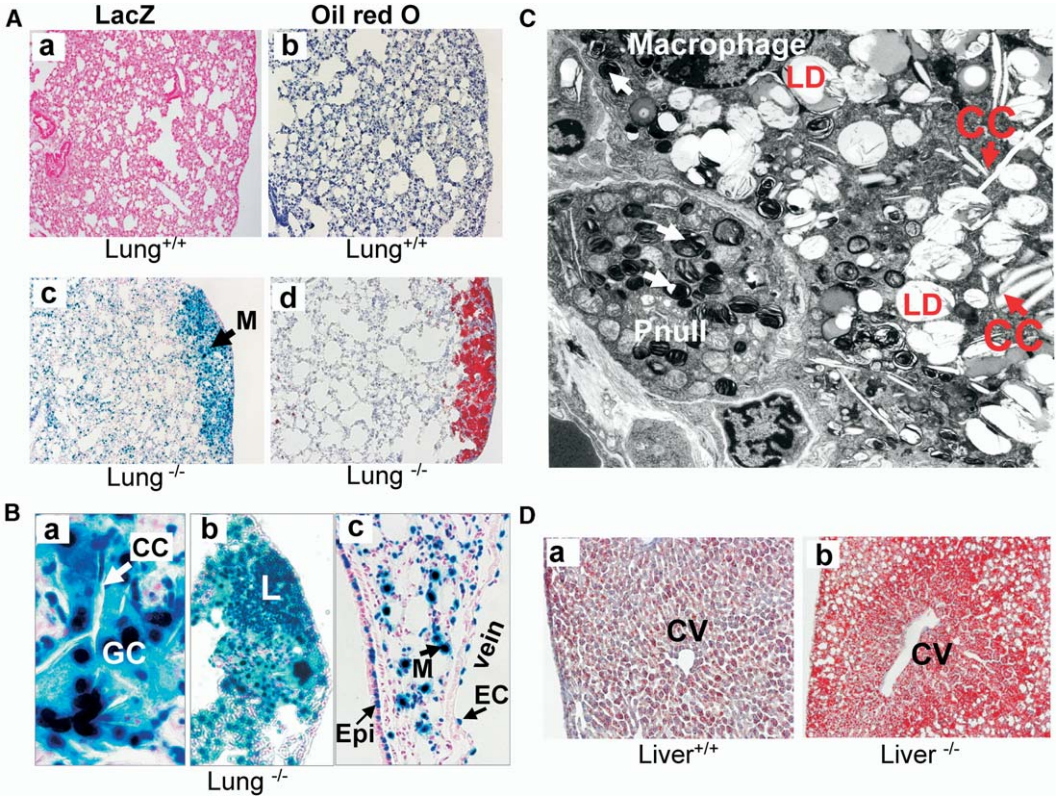

Figure 3. LacZ expression and lipid accumulation in *Abcg1*-null and control mice fed a high-fat/high-cholesterol diet

A) *Abcg1*$^{-/-}$ and *Abcg1*$^{+/+}$ mice were fed a high-fat/high-cholesterol (HF/HC) diet for 9 weeks. Cryosections of the lungs (panels **a**–**d**) were stained for lacZ (panels **a** and **c**) or for neutral lipid using oil red O (panels **b** and **d**), as indicated.

B) Lungs of the HF/HC-fed *Abcg1*$^{-/-}$ mice contain cholesterol clefts (CC), giant cells (GC) (panel **a**), and lacZ-positive lymphocytes (L) (panel **b**). LacZ-positive macrophages (M), epithelial cells (Epi), and endothelial cells (EC) are also evident (panel **c**).

C) Electron micrograph of a lipid-loaded macrophage present in the subpleural lung area of an *Abcg1*$^{-/-}$ mouse fed the HF/HC diet. A few of the lipid droplets (LD), cholesterol clefts (CC), and lamellar bodies (white arrows) are indicated. A pneumocyte type II (PnuII) cell is shown. Magnification = 15,000×.

D) Cryosections of livers taken from HF/HC-fed wild-type (panel **a**) and *Abcg1*$^{-/-}$ (panel **b**) mice were stained with oil red O to identify neutral lipid. Central vein (CV).

Tissue lipid profiles of *Abcg1*-null and *ABCG1*-transgenic mice

The data of Figure 5 show the lipid profiles of the lungs and livers of wild-type, *Abcg1*-null, and human *ABCG1*-transgenic mice fed chow or the high-fat/high-cholesterol diet for 9 weeks. No significant differences in the cholesterol, triglyceride, or phospholipid levels were observed in these tissues of chow-fed animals (Figure 5). In contrast, cholesterol, triglyceride, and phospholipid concentrations were significantly increased in the livers and lungs of *Abcg1*-null mice fed the high-fat/high-cholesterol diet, as compared to their wild-type littermates (Figure 5).

In contrast to the data with the *Abcg1*-null mice, the levels of total cholesterol, cholesterol esters, and phospholipids were significantly lower in the livers and lungs of the transgenic mice fed the high-fat diet as compared to their wild-type littermates (Figure 5). Thus, the human *ABCG1*-transgene protected these tissues from diet-induced lipid accumulation. Interestingly, plasma lipid levels were similar in *ABCG1*-transgenic, *Abcg1*-null, and their littermates fed the high-fat/cholesterol diet (Figure S2), suggesting that the function of ABCG1 is different from other ABC transporters, such as ABCA1, ABCG5, and ABCG8, that are also activated by LXR.

As noted above, the tissues of young ($\leq$7 weeks of age) *Abcg1*-null mice fed chow showed no evidence of lipid accumulation. However, examination of the lungs of chow-fed *Abcg1*-null mice of $\geq$15 weeks of age indicated the presence of oil red O-positive lipid; by the age of 6 months, the lungs of chow-fed *Abcg1*-null mice contained giant cells, multiple lymphocytes, and oil red O-positive macrophages (Figure S4A–S4C). In contrast, the livers of these *Abcg1*$^{-/-}$ mice and the lungs and livers of their wild-type littermates fed chow showed no evidence of neutral lipid accumulation (Figure S4D and data not shown). Together, these data demonstrate that ABCG1 plays a critical and heretofore unrecognized role in maintaining normal cellular lipid levels in both chow-fed mice, but especially in mice following administration of a high-fat/high cholesterol diet.

Dysregulation of cholesterol biosynthetic genes in *Abcg1*-null animals

Given the critical role for LXR in maintaining proper lipid homeostasis of the liver and the fact that *ABCG1* is a target gene of LXR, we were prompted to examine the regulation of genes involved in both fatty acid and cholesterol biosynthesis in wild-type and *Abcg1*$^{-/-}$ animals. *Abcg1*$^{-/-}$ animals fed a standard

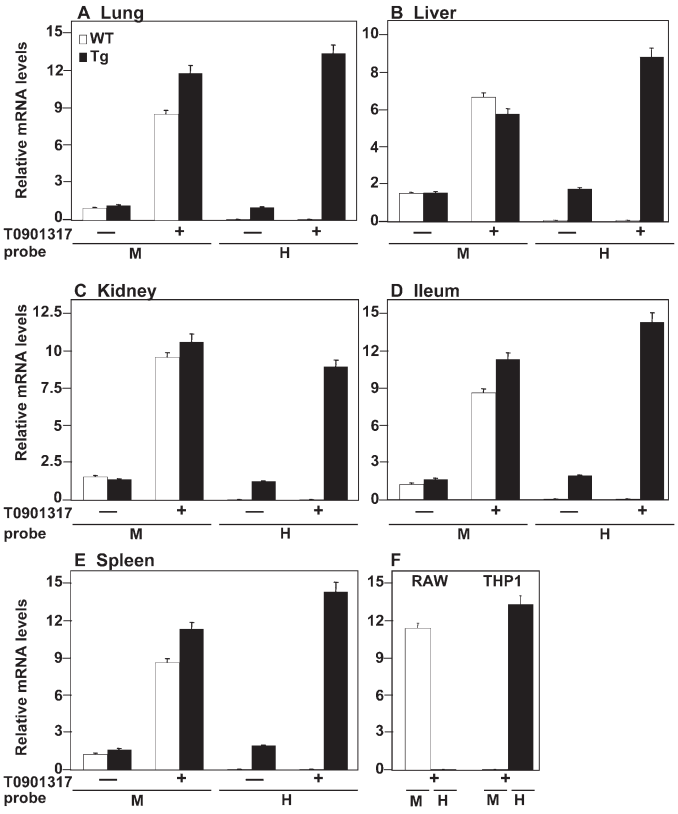

Figure 4. Expression and regulation of the human *ABCG1* in BAC-transgenic mice

Transgenic mice (Tg) and their wild-type littermates (four or five mice/group) were fed chow or chow supplemented with T0901317 (50 mg/kg/day) for 10 days. RNA was isolated from the indicated tissues (**A**–**E**) and murine and human ABCG1 mRNA levels determined using quantitative real-time PCR using probes against the murine (M) or human (H) ABCG1 and normalized to cyclophilin (mean ± SEM, n = 4 or 5 mice/group). ABCG1 mRNA was also quantified from murine RAW267 or human THP1 cells that had been incubated in the presence of T0901317 (1 μM) for 24 hr (**F**).

chow diet displayed a dysregulation of the genes involved in cholesterol biosynthesis and LDL endocytosis; hepatic mRNA levels were significantly increased in the *Abcg1*-null mice (Figure 6A). In addition to the induction of the genes of cholesterol biosynthesis, a 2-fold increase of SREBP1c mRNA was observed in the chow-fed *Abcg1*$^{-/-}$ animals (Figure 6B). SREBP1c was further induced upon feeding animals the high-fat/high-cholesterol diet irrespective of the genotype (Figure 6B). The induction of SREBP1c is consistent with previous studies that demonstrate that in mice fed a high-cholesterol diet, SREBP1c is induced through activation of LXR (Repa et al., 2000). In the presence of excess sterols, LXR activation induces SREBP1c to promote the production of fatty acids that are used as substrates in the esterification of excess free cholesterol by acyl-CoA:cholesterol acyltransferase (Repa et al., 2000). Figure 6C shows that both hepatic acetyl-CoA carboxylase (ACC) and sterol-CoA destaturase (SCD1) were induced ~2-fold and 1.4-fold, respectively, in *Abcg1*$^{-/-}$ mice fed a chow diet. Upon feeding a high-fat/high-cholesterol diet, there was a 5-fold activation of SCD1 independent of genotype and a 2-fold induction of ACC in wild-type mice. Thus, although there is no evidence for lipid accumulation in the livers of *Abcg1*$^{-/-}$ mice on chow, it is clear that there is a dysregulation of the genes involved in cholesterol metabolism. This may account for the activation of SREBP1c and the induction of the genes involved in fatty acid synthesis. An increase in cholesterol levels can also lead to the activation of LXR and SREBP1c and SREBP1c-target genes in an effort to buffer the effect of toxic free cholesterol within the cell (Peet et al., 1998b; Repa et al., 2000). However, under extreme conditions of lipid loading, as is seen when the animals are fed the high-fat/high-cholesterol diet, the genes of cholesterol biosynthesis are repressed and those of fatty acid synthesis, especially SCD1, are highly induced regardless of genotype. Under these conditions, the capacity of the *Abcg1*$^{-/-}$ mice to efficiently process and remove excess lipid is likely defective, leading to the observed increase in tissue lipids.

ABCG1 is required for maximal efflux of cholesterol from macrophages to HDL

From the histological data it is clear that ABCG1 is required to maintain proper lipid homeostasis in macrophages and hepatocytes. Given that ABCG1 was both expressed in macrophages and is a target gene for LXR, we decided to test if the lipid accumulation in the cells of *Abcg1*-null animals was due to a defect in the transport of cholesterol out of the cells to exogenous lipid acceptors. Consequently, peritoneal macrophages were isolated from wild-type and *Abcg1*-null mice and cholesterol efflux to HDL or apoA1 determined. Consistent with a role for LXR in reverse cholesterol transport, treatment of wild-type macrophages with ligands for LXR and RXR significantly increased the rate of efflux of cholesterol to both HDL and apoA1 (Figures 7A and 7B). However, in contrast to these results, treatment of *Abcg1*-null cells with LXR/RXR ligands failed to stimulate cholesterol efflux to HDL (Figure 7A). This effect was surprisingly specific for HDL given that cholesterol efflux to apoA1 was not affected in the *Abcg1*-null cells (Figure 7B). This defect in *Abcg1*-null cells was not a result of an impairment of the LXR/RXR pathway since treatment of the *Abcg1*-null cells with T0901317 and LG100153 resulted in normal activation of ABCA1 mRNA levels (data not shown).

In parallel studies we measured the rate of cholesterol efflux from *Abca1*-null and control peritoneal macrophages to either HDL_3 or apoA1. The data of Figure 7C demonstrate that cholesterol efflux to HDL_3 increased following treatment of either wild-type or *Abca1*-null cells with T0901317 and LG100153. In contrast, cholesterol efflux to apoA1 was attenuated significantly in both untreated and LXR-activated *Abca1*-null macrophages (Figure 7D). Based on these studies, we conclude that ABCG1, but not ABCA1, has a specific role in mediating the efflux of cholesterol from macrophages to HDL following activation of LXR/RXR. In contrast, ABCA1 functions to transfer cellular cholesterol to lipid-poor apoA1. Very recent studies also demonstrated that cholesterol efflux to HDL was increased following transient transfection of cells with cDNAs encoding ABCG1 (Nakamura et al., 2004; Wang et al., 2004). In preliminary studies, we have failed to observe differences in the efflux of choline-labeled or oleic acid-labeled lipids from *Abcg1*-null and wild-type peritoneal macrophages (data not shown).

Taken together, the current mechanistic studies are consis-

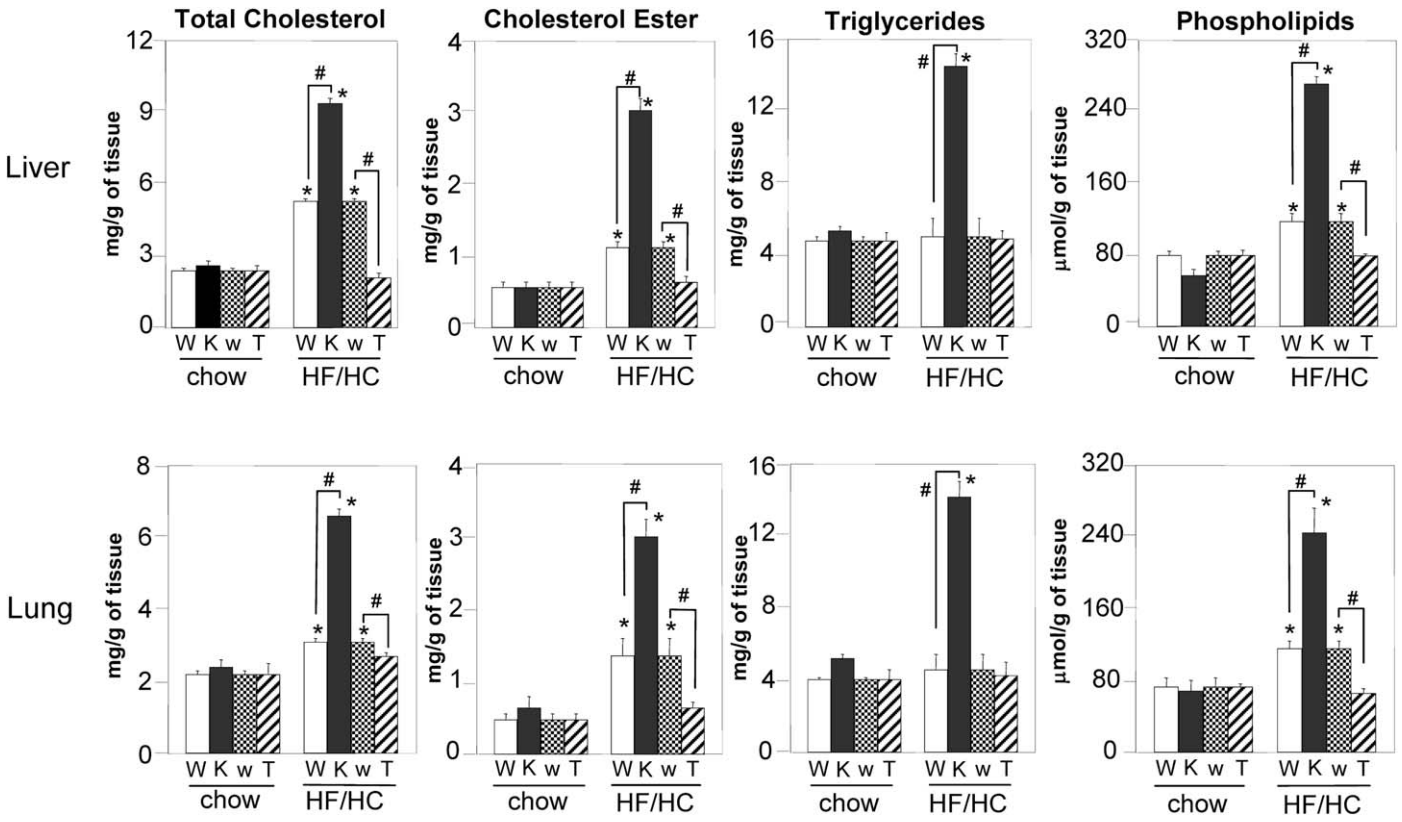

Figure 5. Tissue lipid levels are altered in *ABCG1*$^{-/-}$ and *Abcg1*-transgenic mice

Male *Abcg1*$^{-/-}$ (■; K) and their wild-type (□; W) littermates or transgenic mice (▨; T) and their wild-type littermates (▩; w) (8–9/group) were fed normal chow or the high-fat/high-cholesterol (HF/HC) diet for 9 weeks. Tissues were removed and lipids analyzed as described in Experimental Procedures. Values shown are mean ± SEM, n = 8 or 9.

*, p value (determined by Student's t test) < 0.001 between HF/HC-fed mice and their chow-fed littermates.

#, p < 0.001 between the indicated mice fed the HF/HC diet.

tent with the observed accumulation of lipid droplets in macrophages and hepatocytes of *Abcg1*-null mice and the protective role of human *ABCG1* in transgenic mice.

Discussion

The availability of *Abcg1*$^{-/-}$/*lacZ* knockin and human *ABCG1* BAC-transgenic mice has provided important tools to investigate the cellular and tissue distribution and function of the protein. Young *Abcg1*$^{-/-}$ mice were indistinguishable from wild-type littermates when fed a normal chow diet. In contrast, the lungs of older *Abcg1*-null mice (15–24 weeks) accumulate neutral lipid, multinucleated giant cells, and lymphocytes (Figure S4). However, when young *Abcg1*$^{-/-}$ mice were challenged with a high-fat/high-cholesterol diet, this lipid imbalance was greatly accelerated; the *Abcg1*-null mice failed to control cellular lipid levels and accumulated massive amounts of cholesterol, triglycerides, and phospholipids in multiple organs, without changes in plasma lipids and lipoproteins. Consequently we conclude that ABCG1 has (1) a critical role in regulating lipid homeostasis in multiple tissues and (2) no significant role in regulating the levels of plasma lipoproteins.

In an earlier study, it was reported that plasma HDL and cholesterol ester levels were reduced following infusion of mice with adenovirus expressing ABCG1 (Brewer and Santamarina-Fojo, 2003). Such changes in plasma lipids may result from inappropriately high overexpression of ABCG1 in hepatocytes, a cell type that normally expresses low levels of ABCG1 (Hoekstra et al., 2003). The current observation that massive tissue lipid accumulation occurs in the *Abcg1*-null mice in the absence of changes in plasma lipid levels may be relevant to clinical disease.

Previous studies reported that either cholesterol or oil red O-positive stained lipids accumulate in the liver, brain, and/or macrophages of *Lxrα*$^{-/-}$ or *Lxrαβ* double knockout mice (Alberti et al., 2001; Peet et al., 1998a; Tangirala et al., 2002). Such changes in lipid levels likely result from altered expression of multiple genes. The current study identifies ABCG1 as an important LXR target gene that plays a critical role in maintaining lipid homeostasis in a variety of cells and tissues. Other LXR-target genes that contribute to changes in lipid levels include ABCA1, ABCG5, and ABCG8 since disruption of the latter three genes results in altered plasma lipid levels (McNeish et al., 2000; Yu et al., 2002) and accumulation of cholesterol (or oil red O staining) in the lungs, liver, and/or brain (McNeish et al., 2000; Wang et al., 2002; Yu et al., 2002).

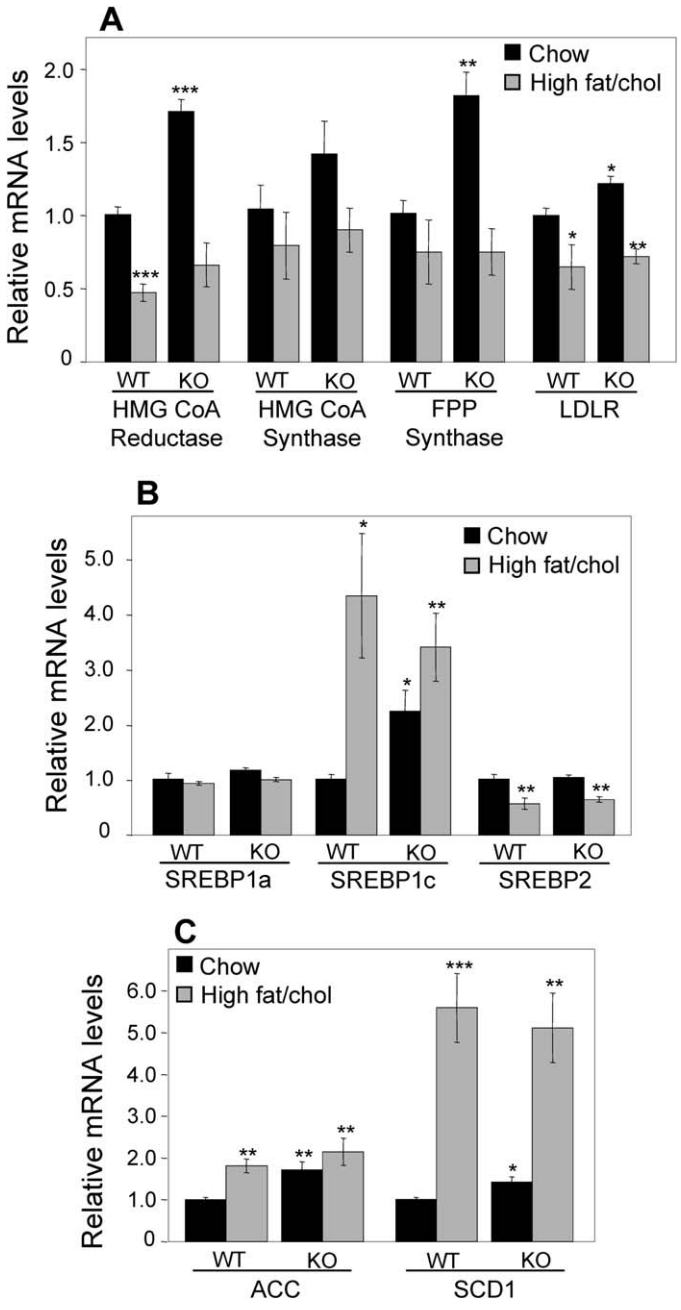

Figure 6. Dysregulation of hepatic genes involved in cholesterol and fatty acid synthesis in *Abcg1*$^{-/-}$ mice

Wild-type (WT) and *Abcg1*$^{-/-}$ (KO) mice were fed chow or the high-fat/high-cholesterol diets for 9 weeks prior to isolation and quantification of hepatic mRNA levels using quantitative real-time PCR. Wild-type and *Abcg1*$^{-/-}$ on chow, *Abcg1*$^{-/-}$ on HF/HC (n = 5 per group), wild-type on HF/HC (n = 4). Values shown are mean ± SEM. mRNA expression in the wild-type mouse fed chow was arbitrarily designated as 1.0. *p < 0.05; **p < 0.01; ***p < 0.001 for experimentals versus chow-fed wild-type mice. p values were determined using Student's t test.

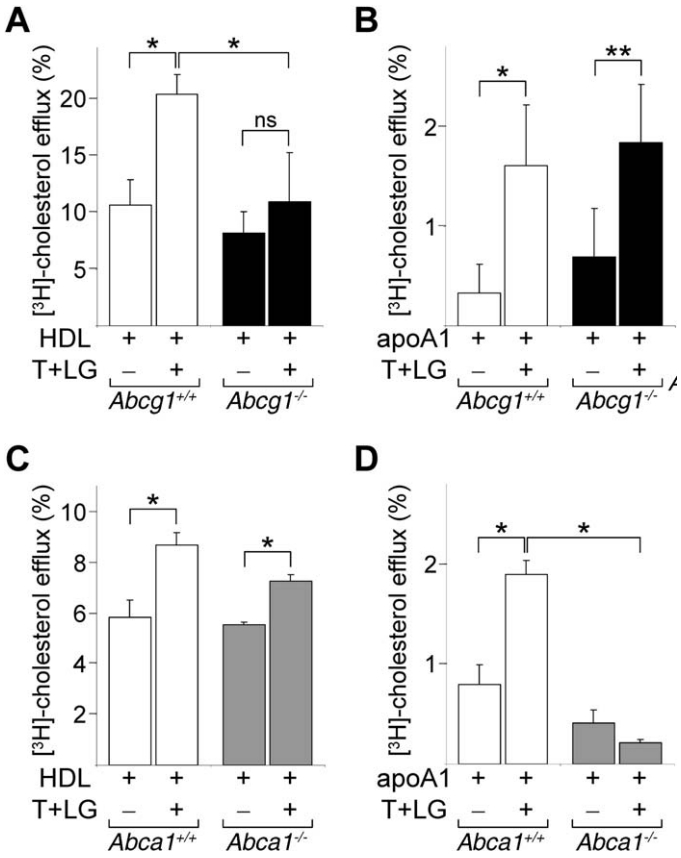

Figure 7. Lipid efflux from peritoneal macrophages to HDL is impaired in *Abcg1*$^{-/-}$ but not in *Abca1*$^{-/-}$ cells

Pooled peritoneal macrophages were isolated from three to seven mice of the same genotype and then incubated in quadruplicate in the presence or absence of LXR/RXR ligands (1 μM T0901317; 100 nM LG100153). Cholesterol efflux was then determined in the presence of either HDL (50 μg/ml) or apoA1 (15 μg/ml), as described in Experimental Procedures. Values are mean ± SD of quadruplicate assays. Data are representative of two or more experiments.
*p < 0.01; **p < 0.02; ns, not significantly different by Student's t test.

The relative importance of ABCG1 and ABCA1 in promoting lipid efflux from macrophages and thus attenuating foam cell formation in vivo is unknown. We hypothesize that the relative concentration of HDL and apoA1 in the sub-intimal space of the artery wall may be important. If, as seems likely, HDL levels are far greater than lipid-poor apoA1 levels, then ABCG1 may play an important role in mediating cholesterol efflux from macrophages and preventing cholesterol ester accumulation and foam cell production. Studies to delineate the importance of ABCG1 in this process in vivo are currently underway. Since the cellular localization of ABCG1 is not known, it remains to be determined whether ABCG1 directly facilitates the transfer of cellular cholesterol from the plasma membrane to HDL or indirectly affects this process by modulating intracellular lipid traffic.

Finally, our data indicate that the absence of ABCG1 results not only in the accumulation of cholesterol esters in macrophages and the liver but also in the accumulation of triglycerides and phospholipids. Identification of the molecular mechanism that results in the accumulation of these latter lipids in the tissues of *Abcg1*-null mice may provide an as yet unknown link between ABCG1 and overall lipid metabolism.

Experimental procedures

Materials

ApoA1 was from Intracell. Bovine serum albumin and X-gal(5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) and ^{3}H-cholesterol (Sigma; 56 Ci/

mM) were from Sigma. Human HDL (d = 1.063–1.21) was isolated by ultracentrifugation. T0901317 was from Cayman Chemicals, Ann Arbor, Michigan.

Animals

All animals were housed under 12 hr/12 hr light/dark conditions. *Abcg1*-null/*LacZ* knockin mice were generated by Deltagen Inc., California, using a targeting vector that contained a 7-kb IRES-*LacZ*-*Neo*-pA cassette flanked by 7 kb of 5′ and 1.4 kb of 3′ mouse genomic DNA. Homologous recombination between the vector and the wild-type *Abcg1* allele results in deletion of 21 bp, encoding seven amino acids (GPSGAGK), that comprise the Walker A motif in exon 3. Chimeric mice were produced from embryonic stem cells derived from the 129/OlaHsd mouse substrain. F1 mice were generated by breeding with C57Bl/6 females, and subsequent generations were crossed once with BABL/cJ mice before multiple (5) back crossing with C57Bl/6. Homozygote (–/–) mice were obtained by crossing heterozygote (+/–) animals.

Human *ABCG1* BAC-transgenic mice were generated at UC Irvine, California, following standard procedures using a 145 kb purified BAC (clone KB1430A10). This clone contains 100 kb corresponding to the full-length human *ABCG1* gene (unpublished data) on chromosome 21 plus additional nucleotides corresponding to 35 kb 5′ and 10 kb 3′ of the gene. Briefly, the BAC was isolated by CHEF gel analysis and purified for injection using Qiagen maxi Prep kit, followed by cesium chloride purification and ultracentrifugation through an ultrafree filter. The purified BAC fragment was quantified using agarose gel electrophoresis, and sets of 144 C57BL/6 eggs were injected with 20 ng of the purified DNA. Twelve pups were genotyped with DNA extracted from tail pieces, followed by subsequent PCR amplification of the 5′ UTR, individual exons 1–23 (Kennedy et al., 2001), and the 3′ UTR of the *ABCG1* gene, and flanking region. Three founder mice were obtained. Southern blot and PCR analysis demonstrated that each founder contained a single insertion of the BAC. Offspring from each founder expressed the human ABCG1 mRNA in multiple tissues. F1 mice were generated by breeding with C57BL/6 females. Subsequent generations were crossed once with BABL/cJ mice before multiple (4) backcrossing with C57BL/6. One line was chosen for more detailed studies.

Diets

Mice were fed for nine weeks either normal chow or a high-fat/high-cholesterol diet (Research Diet; Clinton/Cybulsky D12108; contains 21% fat and 1.25% cholesterol, w/w). Alternatively, mice were fed normal chow supplemented with T0901317 for 10 days (250 mg T0901317 per kg diet) to provide ~50 mg T0901317/kg body weight/day.

RNA isolation and analysis

Tissues, collected from mice fed a chow or high-fat/high-cholesterol diet, were homogenized in Trizol reagent (Invitrogen) for RNA isolation. cDNA was generated from 5 μg of DNase1-treated RNA using Superscript II and Random hexamers (Invitrogen). Real-time PCR was done with Sybr green reagent (Bio-Rad, iQSybr green supermix), using the MyiQ Real-time PCR detection system (Bio-Rad). Primer sets were taken from Yang et al. (2001) or are available on request. Values were normalized to GAPDH and calculated using the comparative C_T method. For Northern blots, 20 μg of total RNA were resolved on a MOPS/formaldehyde gel and transferred to a nylon membrane and probed with a ^{32}P-labeled 3′ UTR probe against murine ABCG1 and cyclophilin.

Lipid analysis

Plasma lipid levels of *Abcg1*$^{-/-}$ and *ABCG1* transgenic mice and their wild-type littermates were assayed enzymatically, as described (Zhang et al., 2004). Tissue lipids were extracted into $CHCl_3$ by a modified Folch method, resolubilized in water, and quantitated using kits for cholesterol, triglyceride, or phospholipid using the accompanying protocols (Wako Chemicals USA, Richmond, Virginia) (Carr et al., 1993).

Peritoneal macrophages and lipid efflux assays

Peritoneal macrophages were obtained from thioglycollate-treated mice as described (Venkateswaran et al., 2000). Macrophages were plated in 48 well plates (0.25×10^6 cells/well) in media containing 10% FBS and allowed to adhere overnight. The cells were washed and incubated for an additional 24 hr in fresh media, re-washed, and incubated in media A (DMEM + 0.2% BSA) supplemented with an ACAT inhibitor (58–035; 2 μg/ml) and ^{3}H-cholesterol (1 μCi/ml). After 24 hr the cells were washed with PBS and incubated in fresh media A for an additional 4 hr equilibration period. Where indicated, the cells were incubated with T0901317 (1 μM) and LG100153 (100 nM) during radiolabeling and equilibration. To determine cholesterol efflux, the cells were rinsed three times and then incubated for 4 hr in media A supplemented, where indicated, with either HDL or HDL_3 (50 μg/ml) or apoA1 (15 μg/ml). The media was removed, the cells washed with PBS, and the radioactive content of the media and cells determined as described (Venkateswaran et al., 2000). Lipid efflux to the media was linear for >8 hr (data not shown). Cholesterol efflux was determined by dividing the radioactive content of the media by the sum of the radioactivity in the cells and media. The basal efflux (% cholesterol that effluxes to media A) was subtracted from the values obtained in the presence of HDL or apoA1.

LacZ expression and Oil red O staining

Tissues were removed and placed in 4% paraformaldehyde in PBS for 4 hr. Briefly, the tissues were rinsed with PBS and cryoprotected by soaking in 20% sucrose in PBS at 4°C overnight. The tissues were then snap frozen in OCT and stored at –80°C. Ten micrometer frozen sections were prepared on glass slides (Fisher super frost plus) and stored at –80°C. To detect β-galactosidase activity the glass slides were incubated for 12–16 hr at 23°C in the presence of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). The slides were washed three times with PBS/0.1% Tween-20, counterstained with nuclear fast red (unless otherwise indicated), rinsed in water, dehydrated, and mounted under coverslips using a xylene-based mounting media. Alternatively, frozen sections were incubated with oil red O, washed with 60% isopropanol, and counterstained with hematoxylin.

Electron microscopy

Thin section electron microscopy was carried out as described (Tamminen et al., 1999).

In situ hybridization of adult murine tissues

Ten-week-old male C57BL/6 mice were fed a diet consisting of either chow (TD 5001) or chow with 50 mpk synthetic LXR agonist T090137 ad libitum for 10 days. Paraformaldehyde perfusion and fixation of the mice were performed as previously described (Lindsell et al., 1995). Tissues were removed and frozen in OCT (VWR) after soaking for 2 days in cryoprotection buffer at 4°C. Twenty micrometer sections were placed directly on Superfrost slides (Fisher) after cryo-sectioning and thaw-mounted followed by 60 min under vacuum and storage at –70°C until hybridization. Sense and antisense cRNA probes were made from the plasmid pCI-Neo- *Abcg1* containing a 905 bp fragment of the ABCG1 carboxy terminus and 3′ UTR as described above using [α-33P]UTP and the MAXIscript in vitro transcription kit (Ambion). Alkaline hydrolysis of the cRNA probe was performed to yield a final fragment length of 0.3 kb. Hybridization was carried out exactly as described (Lindsell et al., 1995) except that the proteinase K treatment was 10 min and the RNase treatment was 35 min for all tissues. Labeled cRNA probe (5×10^7 cpm) was used per slide in the hybridization, and signal detection on X-ray film was performed for 24 and 72 hr with Kodak BioMax MR film at –70°C. Emulsion dipped slides were stored for 2 weeks at 4°C before exposure. Grains were visualized by bright and dark field microscopy.

Supplemental data

Supplemental data include four figures and are available with this article online at http://www.cellmetabolism.org/cgi/content/full/1/2/121/DC1/.

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