ABC-transporters and lipid transfer proteins : important players in macrophage cholesterol homeostasis and atherosclerosis

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Chapter 2

HEPATIC CELL-SPECIFIC ATP-BINDING CASSETTE (ABC) TRANSPORTER PROFILING IDENTIFIES PUTATIVE NOVEL CANDIDATES FOR LIPID HOMEOSTASIS IN MICE

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ABSTRACT

ABC-transporters play an important role in lipid trafficking. Therefore, hepatic expression patterns of ABC-transporters involved in the regulation of cholesterol metabolism were evaluated. RT-PCR analysis showed that the mRNA expression of 38 ABC-transporters detected in livers of C57Bl/6 mice varied greatly. Although most ABC-transporters were ubiquitously expressed, some members displayed very restricted expression patterns, e.g., ABCA6, A8, B1, B8, B10, B11, C3, D2 and G5/G8 were exclusively (>99%) expressed in parenchymal cells. Interestingly, another 13 ABC-transporters, including ABCA4, A5, A9, A13, B2, B9, C1, C5, D3, D4, F2, G1, and G4 were primarily expressed in Kupffer cells. Although Kupffer cells only contribute to 2.5% of the total liver protein, these 13 genes did contain 9-27% of the total liver expression. Western-type diet feeding (0.25% cholesterol, 15% fat) induced the expression of several primarily Kupffer cell expressed genes, including ABCA5, B9, D3, and D4 (2~3-fold higher), whereas the other ABC-transporters were not significantly changed.
Our findings underscore the importance of cellular localization for studying the regulation of key ABC-transporters in liver cholesterol homeostasis. Furthermore, several novel ABC-transporters, including ABCA5, B9, D3, and D4 were identified as putative novel candidates involved in liver macrophage cholesterol homeostasis.

INTRODUCTION

Transport of cholesterol through the body is an important process in the maintenance of total body cholesterol homeostasis. Different steps that control the delivery and disposal of cholesterol are regulated by ATP-binding cassette (ABC) transporters, which constitute a group of evolutionary highly conserved cellular transmembrane transport proteins that use ATP to generate the energy needed to transport metabolites across membranes.[1] To date, 51 members of ABC transporter family have been identified in mice, which, based on structural similarities, have been divided into seven subfamilies, designated ABC A-G.[2] Structurally, ABC-transporters fall into 2 groups: full-transporters having two similar structural units joined covalently and half-transporters of single structural units that form active heterodimers or homodimers.[2] Four members of the ABC-transporter superfamily have major impacts on lipoprotein metabolism and cellular cholesterol biology: 1) ABCA1, a full-transporter that mediates export of cellular cholesterol, phospholipids, and other metabolites to lipid-poor HDL apolipoproteins such as apoAI.[3] 2) ABCG1, a homodimeric half-transporter that plays an important role in macrophage cholesterol efflux.[4] In contrast to A1, ABCG1 facilitates cellular cholesterol and phospholipid efflux from macrophages to mature HDL, but not to lipid-free apolipoproteins.[5,6,7] 3) ABCG5 and G8, which form heterodimers that restrict intestinal absorption and promote biliary excretion of sterols.[8] Hitherto more than 48 human ABC protein genes have been identified and sequenced.[9] It has been reported that many ABC-transporter genes play a role in the maintenance of membrane lipid bilayer and in the transport of fatty acids and sterols within the body,[2] and mutations of ABC protein genes are causative in several genetic disorders in humans.[10] The human ABCA1 gene is the underlying molecular defect in familial HDL-deficiency syndromes, such as Tangier disease.[11] Interestingly, ABCG1 is highly increased in macrophages from Tangier patients with a defective ABCA1.[12] Mutations in either ABCG5 or ABCG8 cause a rare genetic disorder called sitosterolemia, which is characterized by markedly elevated plasma levels of plant sterols and modest increases in plasma cholesterol.[13,14] The discovery of these mutations supports the importance of ABC-transporters in cholesterol homeostasis.

The process of hepatic cholesterol uptake from serum coupled to intracellular processing and biliary excretion plays a pivotal role in cholesterol homeostasis of the body. The liver contains several different cell types, including parenchymal, endothelial, and Kupffer cells. Parenchymal cells are localized around the bile canaliculi, where they mediate both the uptake and metabolism of cholesterol for biliary excretion and the synthesis and secretion of VLDL and HDL. Hepatic endothelial cells line the sinusoids and thus provide a naturally permeable barrier between the blood compartment and the liver parenchyma. In addition, they function in the removal of modified lipoproteins from the circulation. Kupffer cells, resident macrophages of the liver, are strategically located within the sinusoids, where they function in the removal of bacteria and the clearance of modified lipoproteins. ABC-transporters may exert different physiological functions dependent on the individual cell type in the liver.

As scavenger cells, macrophages ingest modified lipoproteins and damaged cell membranes and thus can accumulate large amounts of cholesterol and oxysterols. Interestingly, cholesterol and oxysterols are inducers for several ABC-transporters, including ABCA1 and G1.[15] It is therefore conceivable that a significant portion of the cholesterol-responsive ABC-transporters may be involved in macrophage lipid homeostasis. However, the exact role of individual ABC-transporters in relation to cholesterol homeostasis remains to be further clarified.
Previously, we have shown that ABCG1, which mediates cholesterol efflux, is primarily expressed by Kupffer cells of the liver in rat,[16] indicating its importance in cholesterol metabolism in macrophages, but not parenchymal cells. In agreement, we recently showed that ABCG1 deficiency induced excessive lipid loading in the macrophage-rich regions of the liver, spleen and lung, suggesting a highly significant role for ABCG1 in the removal of lipid from these organs.[17] In the current study, using real-time PCR, the mRNA expression patterns of ABC-transporters and their cellular localization were systematically investigated to identify novel members involved in specific functions relevant for lipid homeostasis in the liver.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (20-25 g) were maintained on regular chow diet containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK), or were fed on semi-synthetic Western-type diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Witham, UK) for 2 weeks. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Hepatic Cell Separation

Mice (n=6 for chow diet and n=6 for Western-type diet) were anaesthetized and the vena cava inferior was cannulated. Subsequently, the vena porta was ligated and the liver was perfused for 10 min (14 ml/min) with oxygenated Hanks' buffer pH 7.4, containing HEPES (1.6g/l). The perfusion was continued for 10 min with Hanks'/HEPES buffer containing 0.05% (w/v) collagenase (type IV, Sigma) and 1 mM $CaCl_2$. Parenchymal cells were isolated after mincing the liver in Hanks' buffer containing 0.3% BSA, filtering through nylon gauze and centrifugation for three times 10 min at 50 g. The pellets consisted of pure (>99%) parenchymal cells (PC) as judged by light microscopy. The supernatants were centrifuged for 10 min at 500 g in order to harvest the non-parenchymal cells. By the modified method as described previously,[18] the endothelial cells (EC) and Kupffer cells (KC) were isolated at 4 °C by density-gradient centrifugation and centrifugal elutriation (3250 rev./min at 26 ml/min for endothelial cells and 65 ml/min for Kupffer cells). The purity of the cell preparations was checked by peroxidase

staining, which showed that Kupffer and endothelial cells were > 80% and > 95% pure, respectively.

Analysis of Gene Expression by Real-time Quantitative PCR

Total quantitative gene expression analysis of isolated liver parenchymal, endothelial, and Kupffer cells was performed as described.[16] In short, RNA was isolated by the acid guanidium thiocyanate-phenol chloroform extraction method according to Chomczynski *et al.*[19] cDNA was synthesised from 1μg of total RNA using RevertAid™ M-MuLV Reverse Transcriptase. mRNA levels were quantitatively determined on an ABI Prism® 7700 Sequence Detection system (Applied Biosystems, Foster City, CA) using SYBR-green technology according to manufacturers' instructions (Eurogentec, Seraing, Belgium). PCR primers (**Table 1**) were designed using Primer Express Software according to the manufactures default settings. We normalized the results to the endogenous controls, HPRT and β-actin. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of HPRT and β-actin ($Ct_{housekeeping}$) and raising 2 to the power of this difference. The average Ct of two housekeeping genes was used to exclude the possibility that changes in relative expression were caused by variations in the expression of separate housekeeping genes.

Data Analysis

K-means clustering was performed on gene expression profiles (relative expressions in endothelial and Kupffer cells compared with parenchymal cells) derived from the primary real-time PCR analysis (Spotfire software). In detail, for the K-means clustering initialization, a data centroid-based search was used with a maximum of three clusters. Similarity between gene expression profiles was determined using a cosine correlation. Furthermore, hierarchical clustering was used through statistical organization and graphical display. All analyses were made against the average relative expression in parenchymal cells (PC). Samples with log ratios of 0 (ratios of 1.0-equal relative expression with PC) were colored black, positive log ratios with reds of increasing intensity, and negative log ratios with greens of increasing intensity. By this method, relationships were represented by a tree whose branch lengths reflected the degree of similarity between genes (Spotfire software).

Data were presented as means ± SEM. Statistical analyses were performed utilising ANOVA using Graphpad Instat Software. The level of statistical significance was set at $P<0.05$.

Table1 Primers for quantitative real time PCR analysis

Gene	Forward primer 5`->3`	Reverse primer 5`->3`	GeneBank accession
Housekeeping genes			
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG	J00423
B-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA	X03672
Control genes			
CD68	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA	NM_009853
CYP7A1	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC	NM_007824
ABC-transporters			
ABCA1	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC	NM_013454
ABCA2	CTGGCACCTGGGAGAGTCA	TGGCCACCGAGTCCAGAG	NM_007379
ABCA3	CTTGCCGTGGACAGGATC	GAATGTTGTGGTTTTTCCAGCTC	NM_013855
ABCA4	ACAGGTGCTCGGCTGATTCT	GCTGCGGATGGTGTGATG	NM_007378
ABCA5	TCATGGGAGAGGCCGCT	GCGAGAAGGCTATGACGAGGT	NM_147219
ABCA6	AAGTTGCAAATGGTCCCTTCAA	GACAACCAGGAGTCCATTCAATG	NM_147218
ABCA8	GGCATGCAACACCAAGAGG	GATGGTGCGAACAGTCCAAGTA	NM_153145
ABCA9	CTGGGATGGACCCCGAA	CCCGCTCTGTGTTTGTGAAAG	NM_147220
ABCA12	AAACATACTTAAAAGATCAGCACCTGAG	GATGTTTGCGACCCCTCCT	AK036764
ABCA13	GTTGCCCTCTGCATTGTCCT	TGTTGCAGTCTTTTAGCTCCAGTC	NM_178259
ABCB1b	CACAGCCAGCATTTCGATAGG	TGTCCCATACCAGAATGCCAG	NM_011075
ABCB2	TTGCAAGTGATGGAATCTACAACAT	CGAAACACCTCTCTGTGCACA	NM_013683
ABCB3	ATTCGGGTCGTGTAATTGACATC	ATGGCGCTTGCAAAGGC	XM_629964
ABCB4	AGGCAGCGAGAAACGGAAC	TGGTTGCTGATGCTGCCTAG	NM_008830
ABCB6	GCCAGGCACGGCAGAGT	AGGAGCAGGAGACCCAAGCTA	NM_023732
ABCB8	TGAGGCCCTTGGCAATGT	TTGATAGCGTTCCTCCTCCCT	NM_029020
ABCB9	GCCGTTGTCGTCGTTTGC	GTGAAAATACCGCCCCGAATA	NM_019875
ABCB10	CAGAAACGTGCACTTCACATACC	GATGGAAAGACTGAAATCCTGGA	NM_019552
ABCB11	TGGAAAGGAATGGTGATGGG	CAGAAGGCCAGTGCATAACAGA	NM_021022
ABCC1	AAGCAGCCTGTACGGATTGTGT	TCACATCCAACTGGGAACTTCC	NM_008576
ABCC2	GGATGGTGACTGTGGGCTGAT	GGCTGTTCTCCCTTCTCATGG	NM_013806
ABCC3	TCCCACTTTTCGGAGACAGTAAC	ACTGAGGACCTTGAAGTCTTGGA	NM_029600
ABCC5	CTCCTCCCACTCCAGTATACAGAACT	TGCCCCTGGTAGCCCTCT	NM_013790
ABCC6	TCCCAAGCTCCTCAGTCTGTTT	GCTAGGAGCCAGCCTGTCC	NM_018795
ABCC8	CGCACAGGCAGTGGGAA	TGCGCCCTTCGAACATG	NM_011510
ABCD1	TGGCCTGGTGGTGTTCCT	TGCCACTAGCTCCCCAAACT	NM_007435
ABCD2	ACCATAGCAAGCGTGGAGGTAA	CATCAATAACTGTTCCTTTGATGGC	NM_011994
ABCD3	TCGTCGGGTACCTGGTTGTC	GCTGTGAAGGTGGCGAGG	NM_008991
ABCD4	TTCTGCGGACGATGAGAGG	TTGCCACCAAGCTGGACA	NM_008992
ABCE1	ATCAGTCCCAAATCAACAGGAAG	ACTGCGGATGCGTGTAAGC	NM_015751
ABCF1	AGGTGGTGGCTGATGAAACAC	TAGCAACTTCAGTCGCTTGGTAT	NM_013854
ABCF2	GCCATGGCAGTGCCAAG	GGATGCCATCATTTTCTGTAGTGT	NM_013853
ABCF3	GGCCTTAGCCCGAGCTCT	CAGCATGTTTGTGGGTTCGT	NM_013852
ABCG1	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG	NM_009593
ABCG2	CACCAGCTCCGATGGATTG	GAGCAACAGAAGCTTGAGGGTT	BC053730
ABCG4	CCGAGACCAGCCGCTTC	TCCCAAAGACTGGGCAACTAAG	NM_138955
ABCG5	TGGCCCTGCTCAGCATCT	ATTTTTAAAGGAATGGGCATCTCTT	NM_031884
ABCG8	CCGTCGTCAGATTTCCAATGA	GGCTTCCGACCCATGAATG	NM_026180

RESULTS

Complete Expression Panel of ABC-transporters in Mouse Liver

To provide a full overview of expression profiles of ABC-transporter mRNAs, real-time PCR was performed, and data analysis was carried out as described in the *Materials and Methods.* Of the analyzed ABC-transporters, 38 were found to be expressed in the liver. As depicted in **Fig. 1**, the hepatic transcript abundance of the detected ABC-transporters varies greatly. Based on the relative expression from the total liver, we classified them into three classes (Class I, II, and III). The relative expression of ABC-transporters in class I is >10. In this class, an extremely high expression of ABCB4 (1355±430) was observed. In addition, ABCG5 (147±49), A6 (107±36), B11 (103±34), D3 (69±16), C2 (42±14), A1 (27±8), D4 (18±4), and A5 (14±3) were included in Class I. ABC-transporters with a comparatively low relative expression <1 were classified into Class III, which was comprised 9 genes, including ABCC8 (0.71±0.20), D2 (0.63±0.21), A9 (0.62±0.17), A4 (0.50±0.12), G1 (0.47±0.04), C1 (0.24±0.05), B1 (0.15±0.05), B9 (0.09±0.02), and G4 (0.07±0.01). In class II, another 20 members displayed medium levels of relative expression (1~10).

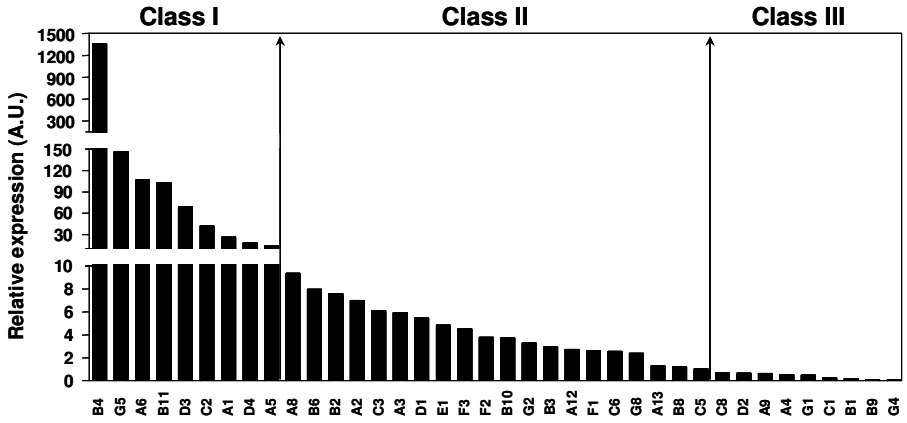

Fig. 1. Complete Expression Panel of ABC-transporters in C57Bl/6 mouse liver on chow diet. Real-time quantitative PCR was used to determine ABC-transporter expression in total liver of C57Bl/6 mice and values were expressed relative to HPRT and β-actin expressions. Based on their relative expressions, the 38 analyzed ABC-transporters were classified into three classes (Class I, II, and III). In class I, an extremely high expression of ABCB4 was observed. In addition, ABCG5, A6, B11, D3, C2, A1, D4, and A5 were included with a high relative expression value (>10). Class III contains transporters with a quite low relative expression value (<1), and comprised 9 genes, including ABCC8, D2, A9, A4, G1, C1, B1, B9, and G4. In class II, another 20 members were included that displayed medium levels of relative expression.

ABC-transporter Gene Expression Profiles of Individual Liver Cell Types

The liver contains several different cell types, including parenchymal, endothelial, and Kupffer cells. Parenchymal cells account for 60% of all liver cells, and >90% of liver protein, while Kupffer cells and endothelial cells only account for 10% and 15% of total cell population, and 2.5% and 3.3% of liver protein, respectively.[16] Low ABC-transporter gene expression in the

total liver thus does not necessarily indicate that this ABC-transporter does not play a role in either the Kupffer cells or endothelial cells of the liver.
Therefore, to gain insight into the cellular localization of ABC-transporters, mRNA was isolated from liver parenchymal (PC), endothelial (EC), and Kupffer cells (KC) from C57Bl/6 mice on chow diet (n=6), and gene expression of the 38 ABC-transporters that were found to be expressed in the liver as well as 2 control genes, CYP7A1 and CD68, were determined. Subsequently, using K-means clustering, three gene clusters were identified based on the similarity of their expression patterns. K-means cluster I (n=17), II (n=8), and III (n=13) contained 38 selected ABC-transporters as well as CYP7A1 and CD68. As shown in **Fig. 2**, Cluster I contained ABC-transporter genes that were mainly expressed in PC, including ABCA1, A3, A6, A8, B1, B3, B4, B8, B10, B11, C2, C3, C6, E1, G5, G8, and D2. For this cluster, CYP7A1, which is solely expressed by PC was included as control. The CYP7A1 expression in PC was more than 100-fold ($p<0.001$) higher than the expression levels found in EC and KC (**Fig. 3a**). Genes ubiquitously expressed in all three cell types were included in cluster II, including ABCA2, A12, B6, C8, D1, F1, F3, and G2 (Fig. 2). Cluster III consisted of 13 ABC-transporters, including ABCA4, A5, A9, A13, B2, B9, C1, C5, D3, D4, F2, G1, and G4, whose expression was much higher in KC as compared to PC and EC (**Fig. 2**). As control for Kupffer cell expression, CD68 which is exclusively expressed in macrophages was included in this cluster, CD68 expression was ~10-fold ($p<0.01$) higher in KC compared with PC and EC (**Fig. 3b**). Intriguingly, although Kupffer cells only contribute to 2.5% of the total liver protein, the above mentioned 13 ABC-transporters in cluster III do contain 9-27% of the total liver expression.

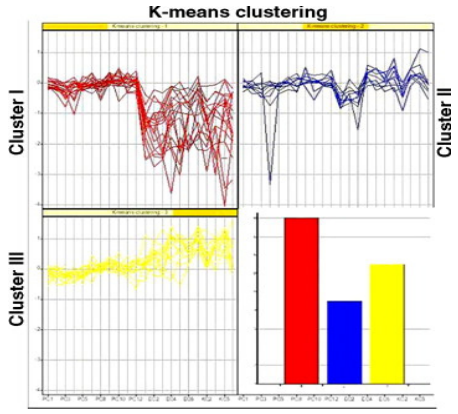

Fig. 2. Gene clusters of ABC-transporters in liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells from C57Bl/6 mice on chow diet. K-means clustering of genes was performed based upon the similarity in their expression profiles. Cluster I (n=17, in red) contains ABC transporter family members that are mainly expressed in parenchymal cells (PC), including ABCA1, A3, A6, A8, B1, B3, B4, B8, B10, B11, C2, C3, C6, E1, G5, G8, and D2. Cluster II (n=8, in blue) contains genes ubiquitously expressed in all three cell types, including ABCA2, A12, B6, C8, D1, F1, F3, and G2. Cluster III (n=13, in yellow) consists of 13 ABC-transporters, including ABCA4, A5, A9, A13, B2, B9, C1, C5, D3, D4, F2, G1, and G4, whose expression is much higher in KC as compared to PC and EC. Three separate clusters are also indicated by bars on the bottom right.

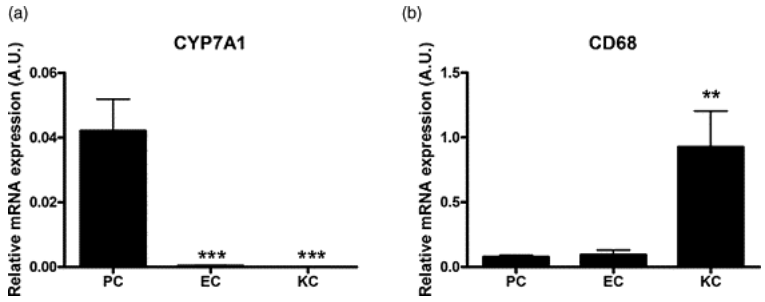

Fig. 3. Relative CYP7A1 (a) and CD68 (b) mRNA expression levels as determined by real time quantitative PCR in liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells from C57Bl/6 mice on chow diet. Values are expressed relative to HPRT and β-actin expressions (mean ± SEM). $p<0.01$ (**) and $p<0.001$ (***) vs PC expression.

In order to assimilate and explore the data in a natural intuitive manner, we also used the hierarchical clustering method. Each data point is represented with a color that quantitatively and qualitatively reflects the original experimental observations. The color scale ranges from saturated green to saturated red, indicating low to high expression against the average relative expression in parenchymal cells. As depicted in **Fig. 4**, three branches (I-III) were identified, using the hierarchical clustering method, which correspond to cluster I-III of the K-means clustering.

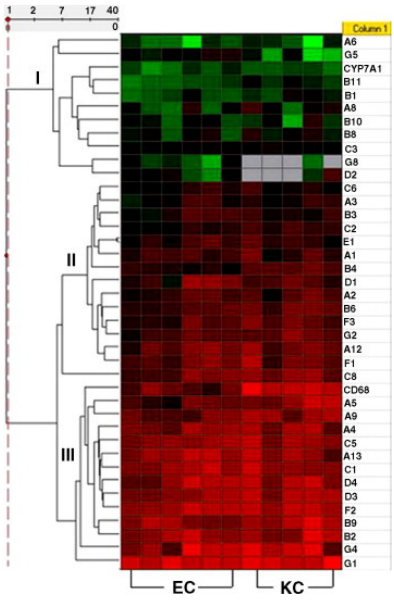

Fig. 4. Hierarchical clustered display of ABC-transporter expression in endothelial (EC), and Kupffer (KC) cells relative to expression in parenchymal cells (PC) from C57Bl/6 mice on chow diet. The colored image was produced as described in Material and Method. Each gene is represented by a single row of colored boxes and each sample is represented by a single column. The color scale ranges from saturated green to saturated red, indicating low to high expression against the average relative expression in PC, respectively. Three branches (I, II and III) were identified, which corresponds to cluster I, II, and III of the K-means clustering. Additionally, CYP7A1 and CD68 are used as a control for PC and KC, respectively.

Although most ABC-transporters were ubiquitously expressed, the cluster analyses clearly showed that some members displayed very restricted expression patterns. In our samples, ABCG5 and G8 were exclusively expressed in parenchymal cells, where their expressions were 8-fold ($p<0.01$) and 26-fold ($p<0.001$) higher than in non-parenchymal cells, respectively (**Fig. 5a, b**). In addition, ABCA6, A8, B1, B8, B10, B11, C3, and D2 were exclusively (>99%) expressed in parenchymal cells. ABCA1 was highly expressed both in parenchymal and in Kupffer cells (**Fig. 5c**). Furthermore, as previously shown,[16] about 20-fold ($p<0.001$) and 12-fold ($p<0.001$) higher ABCG1 expressions were observed in Kupffer and endothelial cells than in parenchymal cells, respectively (**Fig. 5d**). Besides ABCG1, another 12 ABC-transporters, including ABCA4, A5, A9, A13, B2, B9, C1, C5, D3, D4, F2, and G4 were primarily expressed in Kupffer cells. Of these ABC-transporters, ABCD3, D4 and A5 are highly expressed in total liver (relative expression >10, **Fig. 1**). The high expression of ABCD3, D4 and A5 in total liver despite their restrictive expression in Kupffer cells indicates the high levels of expression in Kupffer cells. In **Fig. 5e, f, g**, expressions of these 3 ABC-transporters in PC, EC and KC are shown. ABCD3 expressions in endothelial and Kupffer cells were 3-fold ($p<0.01$) and 6-fold ($p<0.001$) higher than in parenchymal cells, respectively (**Fig. 5e**). ABCD4 expressions in endothelial and Kupffer cells were 5-fold ($p<0.01$) and 7-fold ($p<0.01$) higher than in parenchymal cells, respectively (**Fig. 5f**). Interestingly, ABCA5 mRNA expression in Kupffer cells was 6-fold higher than in both parenchymal ($p<0.01$) and endothelial cells ($p<0.05$) (**Fig. 5g**).

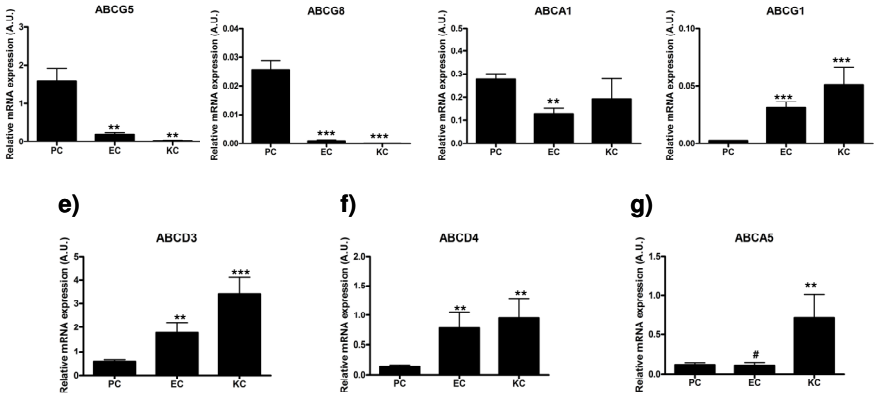

Fig. 5. Relative ABCG5 (a), ABCG8 (b), ABCA1 (c), ABCG1 (d), ABCD3 (e), ABCD4 (f) and ABCA5 (g), mRNA expression levels as determined by real time quantitative PCR in liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells from C57Bl/6 mice on chow diet. Values are expressed relative to HPRT and β-actin expressions (mean ± SEM). $p<0.01$ (**) and $p<0.001$ (***) vs PC expression, as well as $p<0.05$ ($^{\#}$) vs EC expression.

Regulation of Kupffer Cell ABC-transporter Expression by Western-type Diet

To identify ABC-transporters that might be novel candidates for macrophage cholesterol homeostasis, expression profiles of Kupffer cell-expressed ABC-transporters (totally 13 genes in cluster III) in C57Bl/6 mice challenged with

Western-type diet for 2 weeks were compared with those of mice on regular chow diet. Upon Western-type diet feeding, mRNA levels of ABCB9, D3 and D4 were 2~3-fold higher in both endothelial and Kupffer cells (**Fig. 6a, b, c**). Unexpectedly, mRNA expression of ABCF2, a protein with unknown function, was increased ~4-fold ($p<0.01$) specifically in endothelial cells on Western-type diet (**Fig. 6d**).

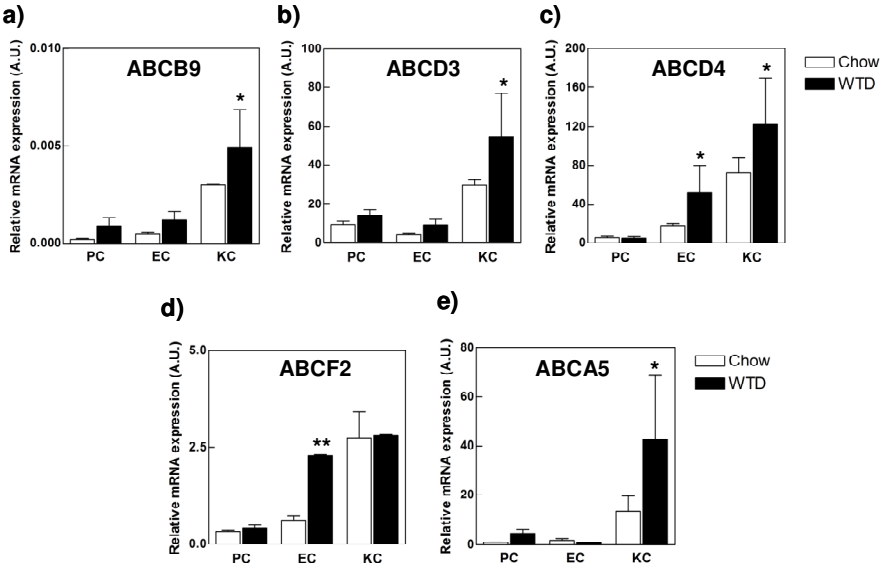

Fig. 6. Effects of Western-type diet on the relative ABCB9 (a), ABCD3 (b), ABCD4 (c), ABCF2 (d), and ABCA5 (e) mRNA expression levels as determined by real time quantitative PCR in liver parenchymal (PC), endothelial (EC), and Kupffer (KC) cells in C57Bl/6 mice. Values from mice on chow diet (open bars) and on Western-type diet (filled bars) are expressed relative to HPRT and β-actin expressions (mean ± SEM). $p<0.05$ (*) and $p<0.01$ (**) vs the relative expression on chow diet.

Another member of the ABC-transporter super family that was highly expressed in Kupffer cells is ABCA5, which belongs to the same ABCA subfamily as ABCA1. In contrast to ABCA1 which is expressed by both parenchymal and Kupffer cells, ABCA5 is primarily expressed in Kupffer cells (**Fig. 5g**). On Western-type diet, ABCA5 mRNA expression increased ~4-fold and ~3-fold ($p<0.05$) in parenchymal and Kupffer cells, respectively, whereas a decreased expression was observed in endothelial cells (**Fig. 6e**). Under this condition, Kupffer cell ABCA5 expression was 10–fold ($p<0.05$) and 51-fold ($p<0.01$) higher than in parenchymal and endothelial cells, respectively (**Fig. 6e**). Other members of Cluster III, including ABCA4, A9, A13, B2, C1, C5, G1, and G4, were not significantly regulated by Western-type diet feeding.

DISCUSSION

Previously, we showed a relatively high expression of the biliary transporters such as ABCG5 and G8 in parenchymal cells compared with endothelial

and Kupffer cells, while ABCG1 was primarily expressed in Kupffer and endothelial cells.[16] In the current study, successfully combining real-time quantitative PCR with cluster analysis, we provided comprehensive data of ABC-transporter expression profiles in mouse liver cells.

In good concordance with earlier reports, cholesterol 7α-hydroxylase (CYP7A1), one of the key enzymes in bile acid synthesis, as well as ABCG5 and G8 are highly expressed in parenchymal cells, which is consistent with their suggested functions. Furthermore, high expressions of ABCB4, B11 and C2 were found in parenchymal cells. ABCB4 is involved in biliary phosphatidylcholine secretion from hepatocytes in a bile salt-dependent manner.[20] Moreover, ABCB4 is required for ABCG5/G8 mediated cholesterol secretion from the hepatocyte into the bile, indicating an indirect role for ABCB4 in cholesterol secretion.[21] Mutations in ABCB4 cause progressive familiar intrahepatic cholestasis-3 (PFIC-3) and are associated with intrahepatic cholestasis of pregnancy.[22] ABCB11 is the major canalicular bile salt export pump for active transport of bile salts across the hepatocyte canalicular membrane into bile.[23] ABCB11 mutations cause PFIC-2, which is an inherited disorder with severe cholestatic liver disease from early infancy.[24] ABCC2 is expressed in the canalicular (apical) part of the hepatocyte and functions in biliary transport of mainly anionic conjugates with glutathione, with sulfate or with glucuronosyl (e.g. glucuronosyl bilirubin).[25] Several different mutations in this gene have been observed in patients with Dubin-Johnson syndrome (DJS), an autosomal recessive disorder characterized by conjugated hyperbilirubinemia.[25]

Modified LDL has been shown to convert macrophages to foam cells. The clearance of modified LDL in vivo is mainly mediated by the liver.[26] Former studies have shown that both formaldehyde-treated albumin and acetylated LDL were primarily taken up via hepatic endothelial cells.[27] Hence, liver endothelial cells may be involved in the protection against the atherogenic action of modified lipoproteins. ABCF2 belongs to the ABCF (GCN20) subfamily, and it encodes a protein of unknown function. Interestingly, our data showed that ABCF2 expression was specifically increased in endothelial cells on Western-type diet feeding, but not similarly induced in parenchymal and Kupffer cells. In addition, ABCD4 mRNA in endothelial cells was also cholesterol-dependent up-regulated. These results may imply a role for ABCF2 and D4 in endothelial cell cholesterol metabolism.

Most interestingly, using ABC-transporter profiling in purified Kupffer cells, we identified putative novel candidates for macrophage cholesterol homeostasis. Owing to their location in the liver sinusoids, Kupffer cells, resident liver macrophages, have been long considered as the major cellular uptake site for modified lipoproteins from the blood circulation. Cholesterol homeostasis in macrophages is of prime importance, as dysregulation of the balance of cholesterol influx and cholesterol efflux will lead to excessive cholesterol accumulation. Previously identified ABC-transporters that facilitate cholesterol efflux from macrophages are ABCA1 and G1.[28,29] In agreement, high relative expression of ABCA1 was observed in Kupffer cells. In addition, a comparably high expression of ABCA1 was found in parenchymal cells, in line with the observation that ABCA1 functions on the basolateral surface of hepatocytes.[30] Previously, we have shown that ABCG1 expression was mainly in non-parenchymal cells in rat.[16]

Comparably, ABCG1 expression was also exclusively expressed in non-parenchymal cells in our samples. Although Kupffer and endothelial cells only contribute 2.5% and 3.3% to the total liver protein, they do contain 27% and 22% of total liver ABCG1 expression, respectively. As previously shown, ABCG1 expression in Kupffer cells was not significantly regulated upon high-cholesterol diet feeding.[16] In addition to these known macrophage ABC-transporters, our data revealed several additional ABC-transporters, including ABCB9, D3 and D4, which are highly expressed in Kupffer cells of the liver and display cholesterol-dependent up-regulation. Members of the peroxisomal half ABCD family play a role in fatty acid beta-oxidation.[31] Mutant ABCD3 (PMP70) knock-out mice have been generated and show abnormalities in the peroxisomal metabolism of bile acid intermediates.[31] Interestingly, in mouse tissues, ABCD3 mRNA expression was highest in the liver and kidney, and was notably induced by fibrates, a peroxisome proliferator-activated receptor α (PPARα) agonist.[31] Fourcade et al showed marked induction of ABCD3 mRNA in the wild type, but not in the PPARα knock-out mouse upon stimulation with fibrate, indicating the fibrate-induced expression of ABCD3 is PPARα-dependent.[32] In addition, ABCB9 and D4 are sensitive to liver-X-receptor and retinoid-X-receptor (LXR/RXR) agonist stimulation.[15] PPARα and LXR are ligand-activated transcription factors that regulate macrophage cholesterol trafficking,[33] implying that ABCD3 as well as ABCD4 and B9 are involved in macrophage cholesterol homoestasis.

Most importantly, we identified ABCA5 as a new transporter which is highly expressed by Kupffer cells. Furthermore, in response to Western-type diet, ABCA5 expression was induced especially in Kupffer cells. The primary expression of ABCA5 in Kupffer cells indicates a potential role of ABCA5 in macrophage cholesterol homeostasis. Like ABCA1, ABCA5 belongs to the ABCA subfamily. Currently, the substrate spectrum of ABCA5, however, is still unknown. ABCA5 is highly expressed in oligodendrocytes and astrocytes of the brain, alveolar type II cells of the lung, and Leyding cells of the testis.[34,35] The pronounced expression in Leydig cells, which is known to process cholesterol and synthesize essential steroid hormones such as testosterone, indicates that ABCA5 may play a role in intracellular sterol/steroid trafficking.[35] Our data indeed showed that ABCA5 expression in Kupffer cells is high and can be further up-regulated by Western-type diet feeding. Klucken et al. have shown that ABCA5 mRNA is up-regulated upon incubation of monocyte-derived macrophages with acetylated LDL and down-regulated upon induction of cholesterol efflux by HDL_3.[36] This regulation was however independent of LXR/RXR stimulation.[15] Recently, ABCA5 knockout have been generated.[34] These mice exhibited symptoms of lysosomal disease in heart, developed dilated cardiomyopathy and died at approximately 10 weeks of age after reaching adulthood. In agreement with the observed lysosomal disease-like symptoms, ABCA5 protein was expressed intracellularly in lysosomes and late endosomes.[34] Additionally, like ABCA5, ABCA1 is also found in the late endosomal and lysosomal compartment and mediates the trafficking of vesicles between these intracellular compartments and the plasma membrane.[37] Furthermore, Tangier disease fibroblasts lacking functional ABCA1 display defective endocytic trafficking, leading to the accumulation of cholesterol in late

endosomes.[38] Thus, ABCA5, like ABCA1, might possibly play a role in macrophage cholesterol homeostasis.
In summary, our quantitative mRNA data for the various cell types are indicative for the activity of the particular genes of interest and their metabolic function. Several novel ABC-transporters were identified, notably ABCA5, B9, D3, and D4 as putative novel candidates relevant for lipid homeostasis in the liver, which may be helpful for further structure/function analysis of currently insufficiently characterized ABC-transporters.

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