Chapter 8

Regulation of hepatobiliary transport and bile formation: lessons from knockout mice

Tineke Kok
Ekkehard Sturm
Folkert Kuipers

Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases,
University Hospital Groningen, Groningen, The Netherlands

Submitted
ABSTRACT

The process of bile formation involves the transport of solutes from blood into bile across hepatocytes by the actions of multiple transport proteins present at the sinusoidal and canalicular membranes. The identification of these transporters and generation of specific knockout mice has greatly contributed to the elucidation of mechanisms involved in bile formation and cholestasis. Mutations in several transporter genes have been identified as the cause of inherited cholestatic liver disorders. In this review, phenotypes of relevant ATP-binding cassette (ABC) transporter knockout mouse models are compared to characteristic hallmarks of the corresponding inherited diseases in humans. Since specific nuclear receptors have been shown to play a prominent role in the transcriptional control of ABC transporter genes, mouse models deficient in nuclear receptors are discussed as far as they affect ABC transporter expression and bile formation.
INTRODUCTION

The formation of bile is an important physiological function of the liver. Bile is essential for the absorption of dietary lipids and lipid-soluble vitamins from the intestine. The biliary pathway represents the major route for elimination from the body of a range of compounds, including bilirubin and xenobiotics. Bile also contains a significant amount of cholesterol, which is important for maintenance of the cholesterol homeostasis\(^1\).\(^3\). Bile formation represents a secretory process that is unique to the hepatocyte. Membrane transport proteins that primarily determine bile formation are located at the basolateral and canalicular membrane domains of hepatocytes\(^4\). Bile salt secretion is a major driving force for the generation of bile flow, which is referred to as bile salt-dependent flow. Other solutes generate the so-called bile salt-independent fraction of bile flow, to which glutathione is a major contributor, at least in rodents\(^5\). After canalicular secretion, bile is modified by cholangiocytes\(^6\) and further concentrated in the gallbladder before reaching the intestine. Several transporters that belong to the ATP-binding cassette (ABC) superfamily of transporters couple ATP hydrolysis to the transport of specific substrates, which enables uphill transport from hepatocytes into the canalicular lumen. In recent years, an impressive progress has been made in the identification and characterization of these transporter proteins. With their identification, a number of inherited cholestatic liver diseases could be ascribed to mutations in genes encoding these ‘pumps’. Cholestatic liver diseases are characterized by an impairment of bile flow and elevated plasma concentrations of biliary constituents, resulting in liver damage. The possibilities to engineer the mouse genome to generate mice that lack these genes (knockout mice) have been extremely helpful in elucidating the roles of the various transporters in physiology and pathophysiology and provide unique models to test potential therapeutic strategies. A summary of hepatic ABC transporters relevant for this review is provided in Figure 1 and Table 1.

In this review, we will focus on hepatic ABC transporter knockout models and compare their features with characteristic hallmarks of inherited (cholestatic) liver diseases in humans caused by mutations in homologous human transporters. The expression of hepatobiliary membrane transporters is known to be regulated both transcriptionally and post-transcriptionally. Several nuclear receptors play a prominent role in the transcriptional control of ABC transporter genes\(^1\)\(^7\). Therefore, this review will also address recent studies showing that absence of nuclear receptors or the presence of specific ligands affects ABC transporter expression and bile formation in mice.

**Figure 1.** Overview of hepatic ABC transporters of which knockout models exist.
Chapter 8

Table 1. ABC transporters (and P-type ATPases) involved in hepatic secretion

<table>
<thead>
<tr>
<th>ABC Transporter name</th>
<th>Species</th>
<th>Gene code</th>
<th>Substrate(s)</th>
<th>Defective in</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSEP Bsep</td>
<td>human mouse</td>
<td>ABCB11</td>
<td>bile salts</td>
<td>PFIC type 2</td>
</tr>
<tr>
<td>MDR3 Mdr2</td>
<td>human mouse</td>
<td>ABCB4</td>
<td>phosphatidylcholine</td>
<td>PFIC type 3</td>
</tr>
<tr>
<td>FIC1 Fic1</td>
<td>human mouse</td>
<td>ATP8B1 (P-type ATPase)</td>
<td>?</td>
<td>PFIC type 1/BRIC</td>
</tr>
<tr>
<td>MRP2 Mrp2</td>
<td>human rat</td>
<td>ABCC2</td>
<td>amphiphatic compounds</td>
<td>Dubin-Johnson syndrome</td>
</tr>
<tr>
<td>ABCG5 Abcg5</td>
<td>human mouse</td>
<td>ABCG5</td>
<td>cholesterol</td>
<td>Sitosterolemia</td>
</tr>
<tr>
<td>ABCG8 Abcg8</td>
<td>human mouse</td>
<td>ABCG8</td>
<td>cholesterol</td>
<td>Sitosterolemia</td>
</tr>
<tr>
<td>MDR1 Mdr1a/1b</td>
<td>human mouse</td>
<td>ABCB1 Abcb1a/b</td>
<td>amphiphatic compounds</td>
<td>?</td>
</tr>
<tr>
<td>CFTR Cftr</td>
<td>human mouse</td>
<td>ABCC7</td>
<td>chloride (bicarbonate)</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>BCRP Bcrp</td>
<td>human mouse</td>
<td>ABCG2</td>
<td>amphiphatic compounds</td>
<td>?</td>
</tr>
<tr>
<td>MRP1 Mrp1</td>
<td>human mouse</td>
<td>ABCC1</td>
<td>amphiphatic compounds</td>
<td>?</td>
</tr>
<tr>
<td>ATP7B Atp7b</td>
<td>human mouse</td>
<td>ATP7B (P-type ATPase)</td>
<td>copper</td>
<td>Wilson disease</td>
</tr>
</tbody>
</table>

ABC TRANSPORTERS, KNOCKOUT MOUSE MODELS AND GENETIC CHOLESTATIC DISORDERS IN HUMANS

BSEP/Bsep
Bile salts are transported across the canalicular membrane by the so-called bile salt export pump (BSEP; ABCB11) in an ATP-dependent fashion. Bsep was identified in 1995 and originally referred to as the Sister of P-glycoprotein (Spgp) on the basis of sequence homology with MDR1 P-glycoprotein (Pgp). Bsep is a ‘classical’ ABC transporter according to the paradigm of 12 membrane-spanning domains and two nucleotide-binding folds. The amino acid sequence of rat Bsep is 49% and 48% identical with rat Mdr1b (Abcb1b) and rat Mdr2 (Abcb4), respectively, and belongs to the B subfamily of ABC transporters. Studies from Gerlof et al., using rat Bsep cDNA expression in Sf9 insect cell line, provided the first convincing evidence that this transporter effectively mediates ATP-dependent bile salt transport. The $K_m$ value for monoanionic bile salt transport by rat Bsep ranges from 2 to 5 $\mu$M, which is in agreement with $K_m$ values of ATP-dependent bile salt transport.
transport in isolated canalicular plasma membrane vesicles. Studies with the cloned mouse Bsep and with human BSEP cDNA also demonstrated high-affinity bile salt transport. The human transporter has similar transport properties compared to its rat and mouse orthologs.

The human BSEP gene is mutated in patients with progressive familial intrahepatic cholestasis (PFIC) type 2, as was demonstrated first by Strautnieks et al. in 1998. PFIC type 2 is characterized by the absence of BSEP from the canalicular membrane and extremely low biliary bile salt concentrations. This supports the notion that BSEP is the major canalicular bile salt transporter in humans. Prominent features of PFIC type 2 are chronic intrahepatic cholestasis, high plasma bile salt concentrations and jaundice. The disease rapidly progresses to hepatic failure requiring liver transplantation before adolescence. Mice deficient in Bsep have been generated in 2001 by Wang et al. Bsep knockout (Bsep(-/-)) mice are cholestatic in the sense that intravenously administered radiolabeled taurocholate accumulates in their liver because its secretion into bile is strongly impaired. However, the mice do excrete substantial amounts of tauromuricholate into bile as well as a tetrahydroxylated bile salt, which has recently been identified as 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid. Bile flow was minimally reduced in Bsep(-/-) mice and, whereas bile salt secretion was reduced to 30% of control values, the secretion of phospholipids and cholesterol into bile was increased. Under normal conditions, the secretion of phospholipids and cholesterol is under the control of bile salt secretion. This is apparently not the case in Bsep(-/-) mice for reasons unknown so far. It might suggest that intrahepatic, rather than intracanalicular, bile salts represent the major driving force for biliary lipid secretion. Another reason could be the upregulation of Mdr2 and/or Abcg5/g8 expression (see below) in Bsep(-/-) mice. This could lead to an inappropriately high phospholipid and cholesterol secretion rate because Mdr2 Pgp activity provides the rate-controlling step in biliary phospholipid secretion and the Abcg5/g8 heterodimer is thought to control biliary cholesterol secretion. No data have been published on this issue so far.

Bsep(-/-) mice clearly do not have the same phenotypic appearance as PFIC type 2 patients. In contrast to humans with a hereditary BSEP defect, targeted inactivation of the Bsep gene in mice results in nonprogressive but persistent intrahepatic cholestasis. The mice hardly show histopathological signs of liver injury. The lower severity of cholestasis in Bsep(-/-) mice may be due to the synthesis of the hydrophobic tetrahydroxylated bile salts, which may neutralize the toxic effect of other bile salts that accumulate in the liver. The fact that bile salt secretion is still possible in these mice, albeit reduced to 30% of normal, may indicate that additional bile salt transporter(s) exist in mice or that other transport systems may be able to handle the differently structured bile salt (conjugates) in Bsep(-/-) mice. Mrp2, the multispecific organic anion transporter (see below), could be a candidate. Partial external biliary diversion is an accepted mode of therapy for PFIC type 2 patients and the majority of patients respond with a significant improvement of symptoms, but the underlying mechanisms thereof remains elusive.

MDR3/Mdr2
The multidrug resistance transporter 3 (MDR3 or ABCB4 in humans; Mdr2 or Abcb4 in rodents) is a phosphatidylecholine translocator predominantly expressed at the canalicular membrane of the hepatocyte. The function of MDR3/Mdr2 was elucidated by disruption of the Mdr2 gene in mice. MDR3/Mdr2 is essential for biliary phospholipid secretion as it translocates phospholipids from the inner to the outer leaflet of the canalicular membrane.
Phosphatidylcholine is disposed from the outer leaflet of the canalicular membrane by a bile salt-stimulated mechanism that is still incompletely understood but may involve vesiculation as well as micellization\(^2\)\(^5\). The secretion of phospholipids is of crucial importance for protection of the cellular membranes of the biliary tree against the high concentrations of detergent bile salts\(^2\)\(^6\).

In the absence of Mdr2 Pgp, mice do not secrete phosphatidylcholine into bile and show a dramatic decrease in biliary cholesterol content\(^2\)\(^1\). Mice heterozygous for Mdr2 disruption (\(\text{Mdr2}^{+/-}\)) show a 40% decrease of biliary phosphatidylcholine output but normal cholesterol output, demonstrating that translocation of phosphatidylcholine is the primary function of the Mdr2 gene product. Mdr2\(^{-/-}\) mice develop portal inflammation and ductular proliferation. Over time, the animals also develop a sclerosing cholangitis and biliary-type cirrhosis and hepatocellular carcinomas. Transplantation of MDR3 transgenic hepatocytes into Mdr2\(^{-/-}\) mice has been shown to restore the normal phenotype and the ability to secrete phospholipids into bile\(^2\)\(^7\).

Elucidation of the genetic background of the PFIC type 3 disease was facilitated by the observation that their phenotypic appearance resembled that of Mdr2\(^{-/-}\) mice\(^2\)\(^8\),\(^2\)\(^9\). Phosphatidylcholine is absent in bile of PFIC type 3 patients and, therefore, bile salts are not associated with phosphatidylcholine in vesicles or mixed micelles. These ‘unshielded’ bile salts are highly toxic to hepatocytes and to cholangiocytes that line the bile ducts. PFIC type 3 patients are characterized by high serum gamma-glutamyltransferase (GGT) activities, high serum bile salt concentrations, a strong bile duct proliferation and development of cirrhosis\(^2\)\(^9\). The most prominent features are portal hypertension, hepatosplenomegaly, jaundice and pruritus. If patients are not treated, the disease will rapidly proceed into liver failure. Bile salt toxicity also appears to play an important role in the development of cholangitis in the Mdr2\(^{-/-}\) mice\(^3\)\(^0\),\(^3\)\(^1\). Feeding of the hydrophilic bile salt ursodeoxycholate (UDCA) to Mdr2\(^{-/-}\) mice improved liver histology significantly, whereas the hydrophobic bile salt cholate was extremely toxic\(^3\)\(^0\). Treatment of PFIC type 3 patients with UDCA appeared to be beneficial in patients with missense mutations, while patients with a truncated MDR3 gene did not improve\(^3\)\(^2\). It seems that the toxic actions of bile salts are more severe in humans than in Mdr2\(^{-/-}\) mice. This is probably due to the more hydrophobic bile salt pool in humans. Therefore, humans probably develop the more severe liver disease earlier in their lives than mice do. MDR3 gene defects do not only give rise to PFIC type 3; increased prevalence of gallstones has been reported in subjects with heterozygote as well as homozygote MDR3 gene mutations\(^3\)\(^2\). Also intrahepatic cholestasis of pregnancy is more frequently observed in family members of PFIC type 3 patients, which turned out to be heterozygotes\(^3\)\(^3\).

**FIC1/Fic1**

The etiology of PFIC type 1, originally referred to as Byler’s Disease\(^3\)\(^4\), is from a mechanistic point of view still poorly understood. PFIC type 1 is caused by mutations in the **FIC1** gene, as revealed by haplotype analysis by Bull *et al.*\(^3\)\(^5\). Mutations in FIC1 are also the cause of a related disease called benign recurrent intrahepatic cholestasis (BRIC). PFIC type 1 manifests itself as a chronic intrahepatic cholestasis. The patients are characterized by high plasma bile salt concentrations, low biliary bile salt concentrations, watery diarrhoea and jaundice. BRIC is a genetic disorder in which patients suffer from periods of cholestasis that are of unpredictable onset and length, and that leaves no liver damage. During the attacks, the patients are severely jaundiced and have pruritus and steatorrhoea. A study by
Bijleveld et al.\textsuperscript{36} has shown that asymptomatic BRIC patients suffer from bile salt malabsorption.

The FIC1 (\textit{ATP8B1}) gene does not encode an ABC transporter, but a P-type ATPase, which is proposed to function as an aminophospholipid translocator\textsuperscript{35}. FIC1 protein is localized at the canalicular membrane of hepatocytes and the apical membrane of cholangiocytes, although the expression in the liver is quite low\textsuperscript{37}. The gene is highly expressed in intestine and pancreas. The function of the \textit{FIC1} gene product remains puzzling, but functional FIC1 seems to be essential for normal bile salt metabolism as mutations are associated with marked disturbances herein\textsuperscript{35}.

Very recently, \textit{Fic1} mutant mice, harbouring the most prevalent missense mutation (G308V) in human PFIC type 1 patients, have been generated\textsuperscript{38}. These mice accumulate bile salts in serum, while the biliary bile salt secretion is maintained at normal rates. This suggests that the primary defect involves dysregulation of intestinal bile salt reabsorption. Because of the limited information published thus far about the \textit{Fic1} mutant mice, we are not able to compare the mice to the human disease. Detailed mechanisms of action of the FIC1 protein remains to be resolved.

\textbf{MRP2/Mrp2}  

The multidrug resistance protein transporter MRP2 (\textit{ABCC2}) is localized in apical membranes of a variety of cell types and is able to transport a wide spectrum of organic anions\textsuperscript{39,40}, including anionic conjugates of glucuronic acid, glutathione (GSH) and sulfate. MRP2 contributes to bile formation by transporting GSH, a major contributor of the bile salt-independent fraction of bile flow\textsuperscript{5}. MRP2 is expressed in liver, kidney and intestine\textsuperscript{41,42}. The liver and the kidney are the major sites for detoxification and excretion of xenobiotics and MRP2 contributes to transport of these substances into bile and urine, respectively. Intestinal MRP2 excretes organic anions into the gut lumen\textsuperscript{43}, probably to promote their fecal disposition.

\textit{MRP2} gene mutations underlie the Dubin-Johnson syndrome\textsuperscript{44,45}. This disease is characterized by a conjugated hyperbilirubinemia, implying that bilirubin can enter the hepatocytes and will be conjugated with glucuronate, but is not secreted into bile and therefore accumulates in the liver or returns back into the blood compartment. Animal models for Mrp2-deficiency are the hyperbilirubinemic Groningen Yellow/TR\textsuperscript{39,46,47} and the Eisai hyperbilirubinemic (EHBR) rat\textsuperscript{48}, which are spontaneous mutants from Wistar and Sprague-Dawley colonies, respectively. The rats have mutations in the \textit{Mrp2} gene that result in a stop codon and premature termination of protein translation\textsuperscript{39}. Mutations in \textit{Mrp2} in rats and humans results in defective excretion of a variety of organic anions, including divalent bile salts, conjugated bilirubin, leukotrienes, as well as many other compounds such as ampicillin, BSP and heavy metals\textsuperscript{49,50}. Mrp2-deficient rat show a decreased bile flow, due to a diminished biliary GSH output\textsuperscript{51}.

A striking similarity between patients with the Dubin-Johnson syndrome and the Mrp2-deficient rats is that hyperbilirubinemia is associated with a dramatic induction of \textit{MRP3/Mrp3} expression in hepatocytes and cholangiocytes. MRP3 (\textit{ABCC3}), like MRP2, is an organic transporter, but is expressed basolaterally. MRP3 is prominently present in the liver, gut and kidney\textsuperscript{52}. MRP3 might play a role in the removal of toxic organic anions from the hepatocyte under cholestatic conditions\textsuperscript{53}. The induction of \textit{MRP3} expression may be considered as a protective adaptation in situations where MRP2/Mrp2 activity is impaired. \textit{Mrp3} knockout mice have recently been generated\textsuperscript{54}, but remain to be
characterized. A difference between the human and rat mutants is that patients have black livers, due to the deposition of a dark pigment in the pericanalicular area, whereas the livers of Mrp2-deficient rats do not show this pigmentation.

**ABCG5/ABCG8 / Abcg5/Abcg8**

ABCG5 and ABCG8 are half-transporters with each having six predicted transmembrane spanning domains. ABC half-transporters are thought to dimerize into functional pumps. Recent evidence suggests that the ABCG5/ABCG8 heterodimer is critically involved in prevention of plant sterol accumulation in the body. Mutations in either ABCG5 or ABCG8 cause the rare autosomal disease sitosterolemia. Although sitosterolemia is not a cholestatic liver disease, ABCG5/G8 is involved in hepatobiliary secretion and therefore discussed in this review. ABCG5 and ABCG8 are highly expressed at the canalicular membrane of hepatocytes and at the apical membrane of enterocytes. Their genes are located oppositely oriented, closely near each other on chromosome 2p21. Recent studies have indicated that ABCG5/ABCG8 in the liver is involved in regulation of biliary cholesterol secretion. Mice overexpressing the human ABCG5/ABCG8 genomic sequence showed elevated cholesterol concentrations in gallbladder bile and enhanced fecal neutral sterol excretion.

Yu et al. demonstrated that Abcg5/g8 knockout mice have a 30-fold increase in plasma concentrations of sitosterol, a major plant sterol. Biliary cholesterol concentrations were strongly reduced, whereas bile salt and phospholipid concentrations were not significantly affected in these mice. Plasma and hepatic cholesterol levels were reduced by 50%. Sitosterolemia patients accumulate large amounts of plant sterols, such as sitosterol, in their circulation. Biliary secretion of sitosterol, cholesterol and other phytosterols is impaired and the absorption of plant sterols in the intestine is strongly increased. The disease is characterized by deposits of cholesterol in their skin, tendon xanthomas, hypercholesterolemia, arthritis and coronary artery disease. The Abcg5/g8 (-/-) mouse phenotypically closely resembles human sitosterolemia in a number of respects. Like the patients, Abcg5/g8 (-/-) mice have an dramatic increase in plasma and tissue levels of plant sterols. Another similarity is that absence of Abcg5 and Abcg8 does not have a detectable effect on the amount or composition of bile salts. Yet, there are also differences between the Abcg5/g8 (-/-) mice and sitosterolemia patients. In mice, plasma cholesterol levels were decreased by 50%, whereas humans have normal or elevated plasma cholesterol levels. Phenotypic differences between the Abcg5/g8 (-/-) mice and sitosterolemic patients may be related to the fact that patients with sitosterolemia usually have defects in a single gene, whereas both genes were disrupted in the mouse model. Knockout models have been created where Abcg5 and Abcg8 are independently disrupted and are currently under investigation.

**Other ABC transporters**

In addition to the previously described ABC transporters, of which most are primarily or secondary affected in cholestatic liver diseases, there are several other transporters that play a role in hepatobiliary transport.

MDR1 (ABCB1) Pgp is able to accommodate a very broad spectrum of amphipathic drugs, thereby conferring multidrug resistance in cancercells. MDR1 Pgp is expressed in many tissues, like the liver, the gut, the blood brain barrier, bone marrow and testis. In hepatocytes, MDR1 is expressed at the canalicular membrane. Two Mdr1 genes (Mdr1a...
Regulation of hepatobiliary transport and bile formation

and Mdr1b) are present in the mouse genome, but only a single MDR1 gene is present in humans. Mdr1a/b knockout mice are hypersensitive to a number of toxic compounds. The mice have a normal bile flow and it has been shown that Mdr1a/b Pgp is involved in the biliary elimination of several cationic drugs\textsuperscript{67-69}. Until now, no disease directly related to MDR1 dysfunction has been identified. Polymorphisms have been detected in the MDR1 gene\textsuperscript{70}, which were found be associated with reduced MDR1 Pgp expression in the duodenum.

The cystic fibrosis transmembrane regulator (CFTR/ABCC7) is a chloride/bicarbonate exchanger, which is localized at the apical membrane of cholangiocytes, but not in hepatocytes. CFTR is activated by secretin via synthesis of cyclicAMP, which induces Cl$$^{-}/$$HCO$$^{3-}$$ exchange that results in increase of bicarbonate and decreased chloride ion concentrations in bile\textsuperscript{71}. A hereditary defect of CFTR is the cause of cystic fibrosis\textsuperscript{72}. CFTR deficiency in humans is associated with impaired ductular bile secretion and cholestasis. Cftr knockout mice display many features common to young human cystic fibrosis patients, including failure to thrive, meconium ileus, obstruction of glandlike structures and alteration of mucous and serous glands\textsuperscript{73}. Studies on bile formation in Cftr knockout mice are currently ongoing in our laboratory.

The breast cancer resistance protein (BCRP/ABCG2) is able to transport a variety of anticancer drugs including topotecan, mitoxantrone and doxorubicin, thus conferring multidrug resistance to cancer cells\textsuperscript{74,75}. Like ABCG5 and ABCG8, BCRP is a halftransporter and consists of a single nucleotide binding fold and 6 transmembrane segments. BCRP is highly expressed in the canalicular membrane of hepatocytes\textsuperscript{76}, but its physiological substrates for hepatobiliary transport are still unknown. No related disease has been characterized until now. Bcrp (-/-) mice develop mild protoporphyria and diet-dependent phototoxicity\textsuperscript{77}.

The multidrug resistance protein transporter MRP1 (ABCC1) functions mainly as a transporter of amphiphatic organic anions, highly comparable with MRP2\textsuperscript{78}. MRP1 is a liver-specific homologue of MRP2, but the transporters have opposite membrane localizations. MRP1 is expressed in most celltypes at the basolateral membrane\textsuperscript{79}. Mrp1 knockout mice have an increased sensitivity to anticancer drugs and show an impaired response to an inflammatory stimulus\textsuperscript{80,81}. During cholestasis\textsuperscript{82,83}, the expression of Mrp1 has been shown to be increased in rat liver.

The transporter ATP7B is, like FIC1, a P-type ATPase which is involved in transport of copper. The protein is predominantly expressed in the liver. ATP7B is localized to the trans-golgi network, when extracellular copper concentration is low. At increased copper levels, ATP7B redistributes to vesicular structures and to apical vacuoles reminiscent of bile canaliculi\textsuperscript{84}, which can be followed by apical (or biliary) excretion of copper. Mutations in the ATP7B gene leads to Wilson disease, a copper toxicity disorder characterized by a dramatic build-up of intracellular copper with subsequent hepatic and neurological abnormalities\textsuperscript{85,86}. Atp7b knockout mice (generated by homologous recombination) and Atp7b-deficient rats, due to a spontaneous mutation in the Atp7b gene, display accumulation of hepatic copper that increases to a level 40- to 60-fold greater than normal\textsuperscript{87,88}. These mice and rats develop cirrhotic liver disease that resembles Wilson disease in humans.
In the past few years, it has become clear that several nuclear receptors play a prominent role in the transcriptional control of ABC transporter genes. Nuclear receptors are small proteins that, upon activation by specific ligands, bind to consensus response elements located in gene promoters to alter gene transcription. Table 2 summarizes nuclear receptors which have been found to play a role in regulation of ABC transporter expression. Most of these receptors belong to the class II of nuclear hormone receptors and require heterodimerization with the retinoid X receptor (RXR) in order to mediate DNA-binding and regulation of transcription. For detailed information about nuclear receptors, the reader is referred to several excellent reviews. In this section, nuclear receptor knockout mouse models will be discussed as far as they affect ABC transporter expression and bile formation.

**PPAR**

Peroxisome proliferator-activated receptors (PPARs) are activated by polyunsaturated fatty acids, eicosanoids and various synthetic ligands. Three PPAR genes have been identified in mammals: PPARα (NR1C1), PPARβ (NR1C2) and PPARγ (NR1C3). PPARα is highly expressed in the liver and is activated by fibrates (hypolipidemic drugs). PPARα is critical in the coordinate transcriptional activation of the fatty acid oxidation machinery in the liver e.g., during fasting. It is becoming clear that PPARα is also involved in control of...
other metabolic pathways, including those of glucose\textsuperscript{96}, amino acids\textsuperscript{97}, and bile salts\textsuperscript{98-100}. This review will address only \textit{Ppar\alpha} knockout mice, because PPAR\alpha plays a role in the regulation of hepatobiliary transporter expression.

\textbf{Ppar\alpha knockout mice}

Studies by Kok \textit{et al.}\textsuperscript{101} demonstrate that, under basal chow-fed conditions, bile formation is not affected by PPAR\alpha-deficiency. Treatment with fibrates increased expression of the phospholipid translocator \textit{Mdr}2 in wild-type, but not in PPAR\alpha-deficient mice, demonstrating that the induction of \textit{Mdr}2 is PPAR\alpha-mediated. Furthermore, 24 hours of fasting, a physiological condition in which PPAR\alpha becomes activated, was also associated with increased \textit{Mdr}2 expression in a PPAR\alpha-dependent manner\textsuperscript{102}. Ciprofibrate treatment of mice led to a massive increase of bile flow\textsuperscript{101}, but the reason hereof is currently unknown. Ongoing studies in our laboratory have revealed that human hepatocytes show increased \textit{MDR}3 expression upon treatment with fibrates (unpublished data), indicating similar modes of regulation of the mouse and human genes.

\textbf{FXR}

In 1999, it was reported that bile salts, such as chenodeoxycholate, deoxycholate, cholate and their conjugates, are high-affinity ligands for the farnesoid X receptor (FXR; \textit{NR1H4})\textsuperscript{103,104}. FXR is highly expressed in the liver, intestine and kidney, i.e., organs that are exposed to bile salts\textsuperscript{105}. The gene encoding the intestinal bile acid-binding protein (IBABP), a 17-kDa bile salt-binding protein\textsuperscript{103,106}, was the first identified target gene of FXR/RXR\textsuperscript{107}. Activated FXR inhibits expression of the gene encoding cholesterol 7\alpha-hydroxylase (\textit{Cyp}7a\textit{\textsubscript{1}})\textsuperscript{108}, which catalyzes the first and rate-controlling step of bile salt synthesis\textsuperscript{109}. This repression is achieved indirectly via a coordinated regulatory cascade involving FXR-mediated induction of the small heterodimer partner (SHP; \textit{NROB}2), which, in turn, inhibits the activity of the tissue-specific factor liver receptor homologue-1 (LRH-1; \textit{NR}5A2), another receptor that controls expression of \textit{Cyp}7\textit{a}1\textsuperscript{108}. FXR also controls the expression of hepatic ABC transporters. Activated FXR induces the expression of the human and rodent canalicular bile salt transporter \textit{Bsep}\textsuperscript{110,111}. Furthermore, a multi-nuclear receptor regulatory element, shared by FXR, PXR and CAR in the promoter of rat \textit{Mrp}2 was reported by Kast \textit{et al.}\textsuperscript{112}. Very recently, FXR was also shown to activate transcription of the human \textit{MDR}3 gene\textsuperscript{113}.

\textbf{Fxr knockout mice}

A first description of \textit{Fxr} knockout mice was provided by Sinal \textit{et al.}\textsuperscript{114} \textit{Fxr}\textsuperscript{(-/-)} mice, on a C57BL/6J-SV129 background, appeared to be very sensitive to a cholate-enriched diet, exhibiting high mortality and hepatic necrosis. Plasma bile salt concentrations were 8-fold increased in the absence of FXR. This was attributed to defective hepatobiliary transport as a consequence of down-regulation of \textit{Bsep} expression. Studies by our group\textsuperscript{115}, using another knockout model of FXR (C57BL/6J-129OlaHsd background), confirmed the downregulation of \textit{Bsep} expression. However, plasma bile salt concentrations were not increased by FXR-deficiency. Surprisingly, despite down-regulation of \textit{Bsep} expression, biliary output rates of bile salts were actually 2-3 fold higher in knockout mice when compared to wild-type controls\textsuperscript{115}. To our opinion, murine Bsep at normal expression levels has a marked overcapacity to secrete bile salts into bile. Other studies by our group\textsuperscript{101} in which we have shown that a strong downregulation of Bsep protein levels in mice was not
associated with impaired biliary bile salt secretion, substantiates this suggestion. Bile flow was slightly increased in FXR-deficient mice, probably due to the increased biliary bile salt output\textsuperscript{115}. Mrp2 expression was unaffected in both published strains of Fxr\textsuperscript{(-/-)} mice\textsuperscript{115,116}. Furthermore, Mrp2 expression was found to be increased in wild-type as well as in FXR-deficient mice during bile salt feeding\textsuperscript{117}, suggesting that FXR regulation of Mrp2 is of limited importance in the *in vivo* situation.

**LXR**

The liver X receptor (LXR) is an important regulator of whole-body cholesterol metabolism\textsuperscript{105}. There are two distinct gene products, LXR\( \alpha \) (NR1H3) and LXR\( \beta \) (NR1H2), which have similar target DNA-binding elements. LXR\( \alpha \) is expressed predominantly in liver, kidney, intestine, fat, spleen and in macrophages, while LXR\( \beta \) is expressed ubiquitously\textsuperscript{105,118}. Natural ligands for LXR are oxysterols, e.g., 22 (R)-hydroxycholesterol and 24 (S)-hydroxycholesterol. It has been proposed that during a high dietary cholesterol intake (via subsequent formation of oxysterols) LXR becomes activated, which, in turn, induces expression of genes involved in cholesterol disposal\textsuperscript{119,120}. Several hepatic ABC transporters have been found to be regulated by LXR, including ABCA1, ABCG5 and ABCG8\textsuperscript{121-123}.

**Lxr knockout mouse**

*Lxr\( \alpha \) knockout, Lxr\( \beta \) knockout as well as double Lxr\( \alpha /Lxr\beta \) knockout mice have been generated\textsuperscript{124-126}. The Lxr\( \alpha \) knockout mice display profound impairments in cholesterol and bile salt metabolism\textsuperscript{124}. By treatment with the LXR agonist T0901317, hepatic and intestinal Abcg5 and Abcg8 expression was increased in wild-type, but not in Lxr\( \alpha /Lxr\beta \) knockout mice\textsuperscript{123}. Studies by Plösch *et al.*\textsuperscript{127} demonstrated that mice treated with the LXR agonist T0901317 have increased biliary cholesterol outputs, possibly related to increased Abcg5/Abcg8 expression. When Abca1 knockout mice were treated with the LXR agonist, biliary cholesterol output was also increased, indicating that this ABC transporter is not involved in biliary cholesterol output, in contrast to suggestions derived from studies in ABCA1 overexpressing mice\textsuperscript{128}. HDL cholesterol is thought to be a major source for bile-destined cholesterol\textsuperscript{129,130}. Despite the fact that HDL is absent in Abca1-deficient mice, biliary cholesterol output is not affected in comparison to wild-type mice and inducible by LXR activation. This suggests that HDL may not be important as a source for bile-destined cholesterol or that its role can easily be taken over by other cholesterol carriers. The bile formation process in the *Lxr* knockout mice has not been studied in detail yet, but is currently under investigation in our laboratory.

**Other nuclear receptors**

Besides the three nuclear receptors described above, there are several other receptors which may be involved in the regulation of hepatic ABC transporter expression.

The nuclear receptor CAR (constitutive androstone receptor) is an obligate RXR\( \alpha \) partner. Unlike the other nuclear receptors, CAR is constitutively active, i.e., a ligand is not required for activity\textsuperscript{131}. CAR is emerging as an important regulator of drug, xenobiotic and bilirubin metabolism\textsuperscript{132}. The *Car* knockout mice exhibit significant impairments in drug metabolism since several members of the CYP2B and CYP3A gene families are CAR targets\textsuperscript{132}. CAR target genes can be activated by phenobarbital, yet, this is not a direct CAR ligand. A multi-nuclear receptor regulatory element, shared by CAR, FXR and
PXR in the promoter of rat Mrp2 was discovered\textsuperscript{112} and Mrp2 is poorly expressed in Car knockout mice\textsuperscript{133}. Rat Mrp3 expression in liver has been shown to be induced by CAR activators\textsuperscript{134}, but studies in Car knockout mice demonstrated that Mrp3 is CAR-independently induced by phenobarbital\textsuperscript{135}.

The pregnane X receptor (PXR) and steroid and xenobiotic receptor (SXR) were discovered in 1998 and soon found to be rodent (PXR) and human (SXR) products of the same gene (NR1I2)\textsuperscript{136-138}. PXR/SXR is highly expressed in the liver and in the intestine. Like CAR, PXR/SXR is an important regulator of drug and xenobiotic metabolism\textsuperscript{139}. Rodent PXR and human SXR have distinct ligand affinities. For example, rodent PXR is activated by pregnenolone 16α-carbonitrile (PCN) and not by rifampicin (RIF), while the human SXR is strongly activated by RIF and not by PCN\textsuperscript{140}. Recent studies suggest that bile salts may also serve as functional ligands for PXR/SXR\textsuperscript{141,142}. Pxr knockout mice fail to upregulate CYP3A mRNA expression with consequent impairments in drug and toxin metabolism\textsuperscript{139,141}. PXR/SXR is an activator of the ABC transporter MDR1\textsuperscript{143} and might regulate Mrp2 transcription\textsuperscript{112}. Treatment of Pxr\textsuperscript{(-/-)} mice with PXR activators induced Mrp3 expression in wild-type but not in Pxr\textsuperscript{(-/-)} mice\textsuperscript{144}, suggesting that PXR plays a role in regulating Mrp3 gene expression. CAR and PXR have been shown to bind to common response elements, showing interaction between the two signaling pathways\textsuperscript{145}.

Three retinoic acid receptor genes have been identified: RARα (NR1B1), RARβ and RARγ\textsuperscript{146-148}, but RARα is the most prevalent isoform in liver and hepatocytes. The liver is the main storage site for vitamin A (9-cis retinoic acid), which stimulates expression and activity of RAR:RXR-dependent genes. Rara knockout mice models mimic phenotypes seen in postnatal vitamin A deficiency\textsuperscript{149,150}, but effects on hepatobiliary transport function have not been reported yet. It has been shown that rat Mrp2 is regulated by the RXR:RAR heterodimer\textsuperscript{151}. The family members of the retinoid X receptor (RXR; NR2B1): RXRα, RXRβ and RXRγ also utilize 9-cis retinoic acid as a high-affinity ligand. RXRα is most highly expressed in the liver and is the obligate heterodimerization partner for several other nuclear receptors. Rxrα knockout mice die in utero of cardiac failure and display significant hepatic developmental abnormalities\textsuperscript{152}.

The orphan receptor small heterodimer partner (SHP, NROB2) was identified in 1996 and its structure revealed a dimerization domain and a ligand-binding domain, but no DNA-binding domain\textsuperscript{153}. No SHP ligands have been identified until now and SHP is able to repress the function of other nuclear receptors. SHP is low expressed in the liver, adrenal, heart and small intestine, and its expression is activated by FXR agonists\textsuperscript{105}. Shp\textsuperscript{(-/-)} mice have been generated\textsuperscript{154,155}, but direct effects on ABC transporter expression and bile formation have not been reported. Monomeric receptors of the hepatocyte nuclear factor family (HNF) have been shown to influence expression of several genes involved in bile salt metabolism and transport by exploiting (liver-specific) knockout mice\textsuperscript{156,157}, but whether this is a reflection of primary regulation or a secondary consequence of disturbed development is not yet clear. It is possible that in the near future mutations in nuclear receptors can be coupled to human diseases. Polymorphisms have already been detected for the PPARα gene\textsuperscript{158,159}. Diabetes and atherosclerosis have been shown to be associated with PPARα polymorphisms but its exact relevance remains to be established.
Transport across hepatocytes from blood into bile involves the actions of multiple transport proteins present at the sinusoidal and canalicular membrane. Progress made in the identification and characterization of hepatic ABC transporters has greatly increased our knowledge of the physiology of bile formation and the etiology of cholestasis. Generation of ABC transporter knockout models has strongly contributed in this respect and have been instrumental in elucidating the genetic background of certain inherited cholestatic liver diseases. Most of the ABC transporter knockout models share phenotypic similarities with patients suffering from inherited cholestatic liver diseases caused by mutations in corresponding human genes. However, as exemplified by the Bsep knockout mouse, these phenotypes are by no means identical. Although data obtained from mice should not be directly extrapolated to the human situation, the ABC transporter knockout models provide useful models to test potential treatment options, including cell transplantation or gene transfer. Transcriptional control of ABC transporter gene expression by nuclear receptors is currently being elucidated at a high pace. This will contribute to a better understanding of the pathogenesis of metabolic liver diseases and may provide novel options for development of new treatment strategies.
Chapter 8


37. Eppens EF, van Mil SW, de Vree JM, Mok KS, Juijn JA, Oude Elferink RPJ, Berger R, Houwen RH, Klomp LW. FIC1, the protein affected in two forms of hereditary cholestasis, is


Chapter 8

spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. Am J Hum Genet 2001; 69:278-90.


81. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC. Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. Cancer Res 1997; 57:5238-42.


91. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell 1995; 83:841-


Regulation of hepatobiliary transport and bile formation


126. Laffitte BA, Repa JJ, Joseph SB, Wilpitz DC, Kast HR, Mangelsdorf DJ, Tontonoz P. LXRa


134. Cherrington NJ, Hartley DP, Li N, Johnson DR, Klaassen CD. Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mmp1, 2, and 3) mRNA and hepatic induction of Mmp3 by constitutive androstane receptor activators in rats. J Pharmacol Exp Ther 2002; 300:97-104.


146. Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to


REFERENCES

20. Verkade HJ, Vonk RJ, Kuipers F. New insights into the mechanism of bile acid-induced biliary