Animal Models of Biliary Disease: Current Approaches and Limitations

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Abstract

Biliary diseases represent an important group of inborn and acquired diseases of the intra- and extrahepatic bile ducts with severe morbidity and mortality due to the development of biliary type of liver fibrosis, liver cirrhosis, and eventually cholangiocarcinoma [1]. The spectrum of cholangiopathies is heterogeneous with respect to underlying mechanisms, clinical course, and presentation. However, these liver diseases share a common target: the cholangiocyte. These diseases include immune-mediated, idiopathic cholangiopathies, such as primary biliary cholangitis (previously known as primary biliary cirrhosis) and primary sclerosing cholangitis, biliary atresia, as well as graft-versus-host disease. The difficulties in studying the complex nature of cholangiocyte injury in humans as well as the currently limited treatment options stress the need for reliable, well-defined, and reproducible animal models in order to gain insights into the pathophysiology and to test novel therapies. The aim of this chapter is to critically discuss the characteristics and limitations of rodent models of biliary diseases for primary biliary cholangitis, primary sclerosing cholangitis, biliary atresia, graft-versus-host disease, as well as cholangiocarcinoma.

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G. Hirschfield et al. (eds.), *Biliary Disease*, DOI 10.1007/978-3-319-50168-0_4

Take-Home Points

- The term biliary disease includes a diverse spectrum of liver diseases, which however share the same primary pathogenetic target: the cholangiocyte.
- The etiology of biliary diseases is unknown, and the use of rodent models is valuable to understand their pathogenesis as well as for testing new drugs.
- A major limitation of the use of animal models is the species differences between rodents and humans.
- Several animal models exhibit comparable characteristics of different biliary diseases.
- There is no "perfect model" that mirrors human biliary disease characteristics making the task of understanding the underlying pathogenetic mechanisms hard. Various aspects of different models might be required to study particular pathogenetic steps.

4.1 Introduction

Biliary disease represents an umbrella term for numerous complex liver diseases which share the primary pathogenetic target within extra- or intrahepatic bile ducts, including primary biliary cholangitis (PBC) (previously known as primary biliary cirrhosis), primary sclerosing cholangitis (PSC), biliary atresia (BA), graft-versus-host disease (GvHD), and cholangiocarcinoma (CCA). However, they differ significantly in regard to their etiopathogenesis, symptoms and clinical course, liver phenotype, gender predominance, and concomitant diseases [1]. The etiology of these diseases still remains only partly understood, but their pathogenesis has become somehow clearer [1–3].

The main advantages for the use of rodent (especially mouse) models are – compared to other mammalians – low costs, the ability for high-throughput studies, ease of handling and breeding, and the possibility of genetic manipulation in mice. Mouse models enable us also to test new drugs and genetic or surgical manipulation within reasonable time frames because of their low life span [4]. However, despite undoubtedly significant research progress, each animal model still harbors its own and important limitations [4]. Such limitations include substantial species differences between rodents and humans in regard to (1) immune and inflammatory responses [5], (2) hepatic and intestinal nuclear receptor expression [6], (3) bile acid metabolism and pool composition [7], and (4) gut microbiota [8] to name only few. In addition, some models exhibit comparable characteristics of different biliary diseases with underlying shared mechanisms of immune-mediated cholangiocyte injury and biliary type of liver fibrosis, and one specific model may consequently mirror different aspects of several cholangiopathies [9]. However, for clarity reasons we would like to provide the characteristics for each model discussed. Due to

limitations of space, we focus exclusively on available mouse models for PBC, PSC, BA, GvHD, and CCA. We would also ask our colleges for pardon, since due to the limitation of references, numerous important publications in this research area could not be cited adequately.

4.2 Primary Biliary Cholangitis (PBC)

The identification of anti-mitochondrial antibodies (AMA) directed against the major mitochondrial antigen pyruvate dehydrogenase complex E2 (PDC-E2) detectable in over 95% of patients leading to consecutive destruction of interlobular bile ducts was a milestone in understanding the autoimmune-mediated nature of PBC [10–12]. Accordingly, PBC represents a classical autoimmune disorder with clear female predominance. Histologically, PBC is characterized by chronic nonsuppurative inflammation with so-called florid duct lesions consisting of epithelioid cell granulomas surrounding small bile ducts eventually progressing to segmental vanishing of bile ducts and ultimately to the biliary type of liver fibrosis and in some patients to liver cirrhosis. Thus, the main attributes of a candidate PBC model include a clear female predominance, presence of AMA in more than 90% and presence of antinuclear antibodies (ANA) in 50–80%, chronic inflammation of small bile ducts with focal duct obliteration and epithelioid cell granuloma formation, chronicity and slow progression of disease with vanishing of bile ducts, biliary type of liver fibrosis with CD4 T cells in liver and hilar lymph nodes, as well as PDC-E2-specific autoreactive CD8 T cells in liver [2].

Animal models for PBC can be subdivided into (1) spontaneous models utilizing genetic modification in mice, (2) neonatally thymectomized mice, (3) inducible models with the use of xenobiotics harboring structural similarities to PDC-E2, and (4) infectious-induced PBC-like phenotype. A list of animal models for PBC is given in Table 4.1. Due to limitations of space, we will focus here on genetically modified mice.

4.2.1 dnTGFβRII Mice

Transforming growth factor-beta (TGF-β) has pleiotropic effects on cell growth and immunological control with a promoting effect on the development of the regulatory T-cell compartment [13]. Overexpression of a dominant negative form of the TGF-β promoter leads to development of PBC-like features with lymphoid cell infiltration of portal fields as well as colitis with 100% AMA positivity [14, 15]. The adoptive transfer of CD8+ cells from these animals into immunodeficient *Rag1−/−* mice underlined the importance of CD8+ cells, since these mice developed similar histopathology to human PBC; however, CD4+ T-cell transfer had no effect on the liver phenotype but worsened colitis [16]. Further studies with an anti-CD20 antibody in young dnTGFβRII showed complete loss of serum AMA positivity and

Table 4.1 Mouse models of PBC

Mouse model	Presence of AMA	Liver phenotype	Limitations/Comments	Reference
Spontaneous models: genetically modified mice				
dnTGFβRII mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury, liver fibrosis	No information on large duct disease; development of colitis	[15–17]
dnTGFβRII IL-12p35−/− mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury, liver fibrosis	No information on large duct disease	[19]
IL-2Rα−/− mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury, large duct disease	No information on fibrosis development, large duct disease; development of colitis	[21]
IL-2Rα$^{-/-}$ IL12-p40$^{-/-}$ mice	n.d.	Lymphoid cell infiltration of portal tracts, bile duct injury	No information on fibrosis development or large duct disease; development of colitis	[22]
NOD.c3c4 mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury, large duct disease	No development of liver fibrosis; large duct disease	[23]
Ae2a,b−/− mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury, liver fibrosis	No information on large duct disease	[28]
Scurfy mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury	No development of liver fibrosis, no information on large duct disease	[30]
MRL/lpr mice	+	Lymphoid cell infiltration of portal tracts	No development of liver fibrosis, no bile duct destruction	[31]
Neonatally thymectomized mice	n.d.	Lymphoid cell infiltration of portal tracts, bile duct injury	No information on large duct disease or liver fibrosis	[120]
Xenobiotic-immunized/infectious-induced models				
2-OA-immunized mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury	No information on large duct disease or live fibrosis	[121]
2OA-BSA-immunized mice + co-treatment with poly I:C	+	Lymphoid cell infiltration of portal tracts, bile duct injury, liver fibrosis	No information on large duct disease	[122]

Mice immunized with LPS + PDH + Freund's adjuvant	n.d.	Lymphoid cell infiltration of portal tracts, bile duct injury, liver fibrosis	No information on large duct disease	[123]
Novosphingobium aromaticivorans-immunized mice	+	Lymphoid cell infiltration of portal tracts, bile duct injury	No development of liver fibrosis, no information on large duct disease	[124]
Escherichia coli-immunized (NOD).B6	+	Lymphoid cell infiltration of portal tracts, bile duct injury, liver fibrosis	No information on liver fibrosis or large duct disease	[125]

Abbreviation: *2-OA* 2-octynoic acid, *Ae2* anion exchanger 2, *AMA* anti-mitochondrial antibodies, *IL* interleukin, *LPS* lipopolysaccharide, *MRL* magnetic resonance imaging, *n.d.* not determined, *NOD* nonobese diabetic, *PDH* pyruvate dehydrogenase, *poly I:C* polyinosinic-polycytidylic acid, *TGF* transforming growth factor

decreased liver inflammation, but were ineffective when initiated in mice with established disease [17]. A central role for natural killer T (NKT) cells in PBC pathogenesis is supported by the generation of CD1d$^{-/-}$-dnTGFβRII mice, in which reduced NKT function caused ameliorated inflammation, bile duct damage, mild ductopenia, cholestasis, and biliary fibrosis [18]. IL-12, consisting of a p40 and a p35 subunit, was studied by generating an *IL-12p35−/−* and *IL-12p40−/−* mouse strain on the dnTGFβRII background [19]. Whereas the *IL-12p40−/−*mice were protected from liver inflammation, in *IL-12p35−/−* mice, liver inflammation with similar severity but delayed onset compared to the parental dnTGFβRII mice was detected [19]. In addition, the deletion of IL-12p35 subunit from dnTGFβRII mice leads to frequent development of liver fibrosis with numerous immunological and histological features similar to human PBC [19]. To further characterize this interesting and promising mouse model, it will be crucial to study the effect of the different cytokines, including IL-12, -23, and -35 on liver phenotypes and on fibrotic changes via cytokine administration or cytokine-neutralizing antibodies [20].

4.2.2 IL-2Rα$^{-/-}$ Mice

Mice with genetic IL-2 receptor deficiency show 100% AMA positivity, lymphocytic portal inflammation, as well as CD4+ and CD8+ lymphocytes infiltrating the bile duct epithelium of intralobular bile ducts [21]. Interestingly, these animals show concomitant severe intestinal inflammation, which is usually not seen in PBC but PSC patients. No hepatic granuloma formation is seen in this mouse model [21]. In addition, there is no information on serum markers for cholestasis and whether also large bile ducts are involved.

Questioning the role of IL-12 in PBC triggered the generation of double knockouts via crossing *IL-2Rα−/−* and *IL-12p40−/−* mice [22]. *IL-2Rα−/−IL-12p40−/−* double-knockout mice show exacerbated autoimmune cholangitis, higher degree of liver fibrosis, and ameliorated colitis compared to *IL-2Rα−/−* single-knockout mice [22]. For more detailed characterization of cholestasis in this interesting mouse model, serum bile acid and alkaline phosphatase levels are awaited [22]. In addition, it would also be important to know whether *IL-2Rα−/− IL-12p-40−/−* mice develop large duct disease.

4.2.3 NOD.c3c4 Mice

The introgression of large genetic intervals on chromosomes 3 and 4 in nonobese diabetic (NOD) mouse strain leads to the development of NOD.c3c4 mice [23, 24]. On histological examination, in a high percentage, eosinophilic infiltration of bile ducts and autoreactivity against the PDC-E2 component are seen. To lower extend destructive cholangitis and granuloma formation can be observed. Whereas these animals show high seropositivity for AMA and ANA (80–90%), unfortunately, we do not have any information on cholestasis parameters of these animals. Intriguingly,

extrahepatic bile duct disease is observed in NOD.c3c4 mice – a feature that would better fit to PSC rather than PBC – with development of cystic dilations of bile ducts, partial exfoliation of the biliary epithelium, and dense neutrophil-granulocytic infiltration [23]. The underlying mechanisms, however, for this peculiar phenotype is not clear and deserves detailed time-course studies (e.g., cholangiography or bile duct plastination for better characterization of large duct disease, characterization of the inflammatory infiltrate). The pronounced neutrophil-granulocytic infiltration of bile ducts could be, at least in part, a secondary phenomenon due to dilatation and secondary ascending cholangitis. Consequently bile culture studies should also be of interest. Interestingly however, treatment of NOD.c3c4 mice with a monoclonal antibody directed against CD3 protected these mice from cholangitis [23]. In general, due to the complex morphological changes in NOD.c3c4 mice, this mouse model may serve as a model for different cholangiopathies, including also several important aspects of PSC pathogenesis.

4.2.4 Ae2a,b$^{-/-}$ Mice

The observations that anion exchanger 2 (AE2) is downregulated in the liver and lymphocytes of PBC patients and that ursodeoxycholic acid restores AE2 expression and stimulates biliary bicarbonate secretion partially by activation of hepatic AE2 [25–27] were the trigger to generate Ae2a,b$^{-/-}$ mice. This mouse model shares some immunologic and hepatobiliary features with PBC [28]. Histologically, mild to severe portal inflammation with high interindividual variations in regard to the liver phenotype is observed. In addition, the defective Treg cell function and CD8+ T-cell expansion seen in these mice could be due to the AE2 dysfunction, which seems to be critically involved also in the homeostasis of the immune system. However, so far a detailed characterization of this model in regard to investigation of large ducts and in regard to potential biliary fibrosis has been not performed. One major limitation of the model may lie within the fact that this mouse strain seems to be very difficult to breed (personal communication Juan Medina, Pamplona, Spain).

4.2.5 Scurfy Mice

Scurfy mice with a selective loss of the transcription factor Fox-P3 (forkhead box P3, also known as scurfin) resulting in a functional deficiency of Treg cells show serological and morphological features of immune-mediated cholangitis, including severe bile duct injury [29, 30]. However, serum bile acid and alkaline phosphatase levels are not reported in these mice. Findings in scurfy mice underline the potential importance of Treg cells for the pathogenesis of PBC. One of the major limitations of this model is based on an extremely short life span of these mice of about 4 weeks, which seriously limits their use for longitudinal studies (e.g., disease progress, drug testing).

4.2.6 MRL/lpr Mice

MRL/lpr mice with the lymphoproliferative gene lpr (also known as MRL/MP-lpr/lpr) spontaneously develop severe autoimmune-mediated disorders, such as vasculitis, glomerulonephritis, inflammation of salivary glands, interstitial pneumonia and plasma-cellular infiltration of portal fields with biliary injury, and development of AMA [31]. The relatively low percentage, about 50% of mice showing PBC-like features, critically limits the usefulness of these mice as a PBC model.

Currently no "ideal PBC model" exists among the available mouse models. Although an enormous progress has been achieved in the last decades in the generation of different model systems that show astonishing similarities with human PBC, concerning immunological and histological characteristics, each model harbors still its specific limitations. As PBC represents a chronic cholangiopathy with slow progression to biliary fibrosis and cirrhosis, long-term studies with detailed characterization of the cholestatic phenotype would be of major interest and urgent need for these models.

4.3 Primary Sclerosing Cholangitis (PSC)

PSC leads to irregular scarring of the biliary tree causing bile duct strictures and dilatation-affecting intra- and extrahepatic bile ducts and may finally lead to biliary cirrhosis and liver failure. PSC primarily affects young men and is frequently associated with inflammatory bowel disease with specific clinical features including rectal sparing, right-sided disease, and backwash ileitis (i.e., PSC-IBD) [32]. The main attributes of an ideal PSC model therefore include the following clinicopathological features: male predominance, progressive fibrous-obliterative cholangitis of medium-sized and large bile ducts, onionskin-type-like periductal fibrosis, biliary type of liver fibrosis, concomitant predominantly right-sided mild colitis or pancolitis, and the high risk for CCA.

Animal models for (primary) sclerosing cholangitis [(P)SC] arbitrarily can be clustered into six different groups [33]: chemically induced cholangitis, knockout mouse models, cholangitis induced by infectious agents, models of experimental biliary obstruction, models involving enteric bacterial cell-wall components or colitis, and models of primary biliary epithelial and endothelial cell injury. Subtypes of models, their respective characteristics, and according references are summarized in Table 4.2. Due to limitations of space, we have to focus on only a few of them.

4.3.1 Mdr2 (Abcb4) Gene Knockout Mice

Mdr2−/− mice show key features of human SC with development of cholangitis and onionskin-type periductal fibrosis similar to human PSC with strictures and dilatations of bile ducts and biliary type of liver fibrosis. Pathogenetically, the lack of biliary phospholipid secretion and increased concentration of free

Table 4.2 Animal models of sclerosing cholangitis

Mouse model	Mice	Liver phenotype	Limitations	Ref.
Chemically induced cholangitis				
DDC	Swiss albino mice PDX-1 knockout mice	Pericholangitis; periductal fibrosis; ductular proliferation; biliary type of fibrosis	No characteristic BD strictures and dilatation on plastination	[34–37]
LCA	Swiss albino mice	Bile infarcts; destructive cholangitis; periductal fibrosis	No tolerable long-term protocol established	[38]
Knockout mouse models				
Abcb4$^{-/-}$	FVB/N	Cholangitis; pericholangitis; periductal fibrosis; biliary type of fibrosis	No colitis or CCA but liver cell tumors	[39–46]
Cftr$^{-/-}$	C57BL/6J	Focal cholangitis; ductular proliferation	High risk for intestinal obstruction, weak spontaneous phenotype (without DSS)	[47–51]
fch/fch	BALB/c	Cholangitis; ductular proliferation; biliary type of fibrosis	Extrahepatic BD not studied so far	[35, 36]
Infectious agents				
Cryptosporidium parvum	BALB/c nu/nu, BALB/c SCID, C57BL76-SCID, NIH-III nu/nu CD40$^{-/-}$, IFNγ$^{-/-}$, CD154$^{-/-}$, CD40-CD154$^{-/-}$, Tnfsf5$^{-/-}$, Tnfrsf1a$^{-/-}$, Tnfrsf1b$^{-/-}$, Tnfrsf1a/1b$^{-/-}$, Tnfsf5-Tnfrsf1a$^{-/-}$, Tnfsf5-Tnfrsf1b$^{-/-}$, Tnfsf5-Tnfrsf1a/1b$^{-/-}$,CD40-Tnfrsf1a/1b$^{-/-}$	Strongly depending on genetic background: cholangitis; pericholangitis; periductal fibrosis; biliary type of fibrosis	Complex models, phenotype so far not well characterized	[52–55]
Helicobacter hepaticus	A/JCr, C3H/HeNCr, C57BL/6NCr, A/J	Cholangitis; pericholangitis	Complex models	[56, 57]

(continued)

Table 4.2 (continued)

Mouse model	Mice	Liver phenotype	Limitations	Ref.
Common bile duct ligation	C57BL/6 J	Bile infarcts; cholangitis; pericholangitis; periductal fibrosis; biliary type of fibrosis	Technical pitfalls	[58, 59]
Models of biliary epithelial and endothelial cell injury				
Experimental GVHD	BALB/c	Cholangitis; pericholangitis; periductal fibrosis; biliary type of fibrosis	Low fibrotic response	[60]

Abbreviation: *CCA* cholangiocellular carcinoma, *Cftr* cystic fibrosis transmembrane conductance regulator, *DDC* 3,5-diethoxycarbonyl-1,4-dihydrocollidine, *DSS* dextran sodium sulfate, *fch* ferrochelatase, *GVHD* graft-versus-host disease, *IBD* inflammatory bowel disease, *LCA* lithocholic acid, *Mdr2* multidrug resistance protein-2

non-micellar-bound bile acids cause damage of bile duct epithelial cells [61] due to regurgitation of bile into the portal tracts leading to inflammation and fibrosis [58, 62]. However, the pathogenetic cause of disease still has to be determined in more detail, especially in regard to the specific role of bile acids. The *Mdr2−/−*mouse model proved to be useful to test novel treatment strategies for (P)SC and liver fibrosis of the biliary type. Hence, this model is increasingly used [39–43, 58, 63–66]. Since the fibrotic response is strongly influenced by the genetic background and varies, it will be interesting to determine the potential effects of mouse genetic background on liver fibrosis degree in *Mdr2−/−* mice. Only male mice should be used for modeling PSC, since female *Mdr2−/−* mice develop gall stone disease already at young age, which is not a common feature in PSC patients and would also lead to significant variations in the cholestatic phenotype of animals [67]. One of the major limitations of this model, however, is the fact that there is insufficient evidence for the impact of MDR3 mutations/dysfunction or low biliary phospholipid output in PSC pathogenesis [68]. In addition, *Mdr2−/−* mice do not develop colitis (at least in the genetic backgrounds tested already) or CCA but hepatocellular neoplastic nodules, which is unusual in PSC patients [69].

4.3.2 Mice Harboring a Mutation of Exon 10 of the Cystic Fibrosis (CF) Transmembrane Conductance Regulator Gene Knockout Mice (*Cftr*$^{-/-}$ Mice)

Cftr−/− mice develop focal cholangitis with inspissated bile and bile duct proliferation, resulting in biliary cirrhosis. Since CFTR gene mutations may play a pathogenetic role in PSC [70], *Cftr*$^{-/-}$ mice proved useful in the study of PSC development, since CFTR gene mutations may play a pathogenetic role in PSC although being not entirely clear so far [47]. A major limitation of this specific mouse model is that the genetic background strongly determines liver and/or intestinal phenotype [48–51].

4.3.3 Mice with a Point Mutation in the Ferrochelatase Gene (fch/fch) and Mice Fed the Porphyrinogenic Substance 3,5-Dietoxycarbonyl-1,4-Dihydrocollidine (DDC)

Both mice show sclerosing cholangitis and pronounced biliary fibrosis paralleled by ductular proliferation and portoportal bridging within weeks [34–36]. However, neither strictures nor dilations of the large duct system despite showing definite histological features of typical periductal fibrosis in PSC are seen which takes 4–8 weeks after DDC feeding depending on DDC-diet concentration and the mouse strain used [34]. The pathogenetic cause of disease is most likely linked to the biliary excreted DDC metabolite protoporphyrin IX and resulting ductal porphyrin plugs [34]. In addition, a link between DDC feeding and interference with biliary phospholipid secretion has been described [37]. The main advantages of this model include high reproducibility, high suitability for pathophysiological studies on the

mechanisms of cholangitis, ductular reaction, and biliary type of liver fibrosis. However, the use for testing of treatment strategies for SC is limited due to the fixed liver phenotype and possible drug-drug interactions.

Taken together similar to PBC, currently there is no "ideal PSC model" available [63, 71, 72]. Since PSC represents a long-standing disease with complex underlying pathogenetic mechanisms, in which endogenous and exogenous factors are involved, it seems not likely that one single model will perfectly mirror PSC, but we will rather need various aspects of different models to study particular pathogenetic steps of PSC.

4.4 Graft-Versus-Host Disease (GvHD)

Bile ducts are major targets in acute and chronic GvHD representing a common complication and limiting factor of an allogeneic tissue and bone marrow transplantation. In humans, acute GvHD occurs within 100 days of transplant, and chronic GvHD (cGvHD) typically develops 100 days after transplantation. In mice, this temporal classification is not necessarily seen, since disease manifestation can differ in time of onset and is mainly defined by the clinical phenotype. Thus, chronic GvHD develops within weeks after transplantation in most mouse models [73]. Pathogenetically, cholangiocytes of small- to medium-caliber bile ducts are the major targets of T-cell-mediated destruction, causing apoptotic cell death and ultimately ductopenia [74]. So far, the detailed pathomechanism of GvHD is not clear.

In mice, GvHD across minor histocompatibility antigens can be induced experimentally by injection of spleen and bone marrow cells of congenic B10.D2 mice into sublethally irradiated BALB/c mice [60]. Bile ducts develop severe cholangitis with predominate lymphocytic inflammatory infiltrates 2–3 weeks after transplantation, and later on periductal fibrosis is observed. The major limitations of this mouse model are that neither loss of intrahepatic small bile ducts nor progression to liver cirrhosis during an observation period of 14 month is observed [60]. Generally speaking, factors confounding the translation of findings in mouse models to the human disease lie behind the fact that in humans acute GvHD typically precedes the chronic form, although in some cases chronic GvHD can occur without the occurrence of clinically obvious acute GvHD [73]. In addition, most patients are given immunosuppressive therapy to prevent acute GvHD influencing the development of chronic GvHD and further complicating modeling human GvHD in animals.

4.5 Biliary Atresia (BA)

BA is the most frequent identifiable cause of neonatal cholestasis, and most patients require early liver transplantation [75]. To date, the underlying pathophysiological mechanisms are unknown, although a pivotal role for a dysregulation of cellular and humoral immunity, viral, toxic, and genetic factors are considered [75]. To date, different model systems for BA have been established, including young lambs and calves

[76], sea lampreys [77, 78], zebrafish [79] and mice [79–83]. In newborn BALB/c mice, infection with rhesus rotavirus type A (RRV) in the first 2 days of life leads to liver disease with development of hepatobiliary injury and cholestasis within 1 week of infection [82, 83] mimicking human BA in several aspects [82–84]. Intriguingly, this mouse model shares major clinicopathological features with the human disease, including a time-restricted susceptibility of bile duct injury to the early postnatal period, acholic stools, bile duct proliferation, and portal inflammation as well as type 1 rich inflammatory infiltrate in the liver and bile ducts [84–90]. However, one of the main limitations of this mouse model is the high mortality rate of mice.

4.6 Cholangiocarcinoma (CCA)

CCA is an epithelial biliary malignancy that originates from oncogenic transformation of cholangiocytes. Depending on the anatomic site, they may originate from different cell types, including intrahepatic biliary epithelial cells, hepatic progenitor cells, or mucin-producing cuboidal cholangiocytes of the extrahepatic biliary epithelium and peribiliary glands [91]. The identification of cellular origin in different subtypes may represent a prerequisite for effective therapy, but its impact on prognosis remains uncertain. CCA carcinogenesis is not entirely clear; however, well-known risk factors include the presence of PSC, liver fluke infections, hepatolithiasis or chronic hepatitis C, cirrhosis and toxins sharing induction of chronic cholestasis, and biliary and/or liver inflammation [92–95]. In the last years, several rodent models of CCA have been developed, including mice with xenograft and orthotopic tumors [96–102], genetically modified CCA models [103–105], and carcinogen-induced CCA models [106, 107]. Although these models provide adequate tools to gain insights into the pathophysiology of CCA development and to test new potential therapeutic agents in a preclinical setting, they harbor important limitations and difficulties discussed below (summarized in Table 4.3).

4.6.1 Xenograft and Orthotopic Models

In xenograft models, CCA cell lines are implanted into nude or severe combined immunodeficiency (SCID) mice. In 1985, the first study of CCA was developed by injecting a cell line derived from a human CCA metastasis subcutaneously into the flank of nude mice [108]. This model has not only been used for performing time-course and pathophysiological studies in regard to tumor growth, but also a high number of potential antitumor agents, including caffeic acid, tamoxifen, melatonin, and clobenpropit, have been tested [110–112]. Currently, although being not entirely clear so far, there is increasing evidence for a pivotal role of MicroRNAs (miRNAs) in cholangiocarcinogenesis with miR-26a and miR-494 known to promote tumor growth via targeting Wnt signaling pathway or modulation of the cell cycle [100, 113]. The informative value of studying interactions between cancer and peritumoral stroma cells in these mice, however, is limited, since tumor growth strongly

Table 4.3 Mouse models of CCA

Model	Mouse	Latency for tumor development	Limitations	Reference
Xenograft	Nude mouse	2 weeks	No metastases	[108]
p53 knock out mouse + CCl_4	$p53^{-/-}$ C57B16 mouse CCl_4 ip at the age of 6 months	$p53^{-/-}$: 29 weeks *p53+/−*: 53 weeks	No information on metastases	[109]
Smad4-Pten knock out mouse	Cre-mediated deletion of Pten and Smad 4	4–7 months	Absence of chronic liver injury and inflammation, no metastases, concomitant of the salivary glands tumors	[104]
DEN + left median bile duct ligation	ip injection of DEN in young Balb/c + left median bile duct ligation + oral gavage of DEN	28 weeks	Model complexity	[107]

Abbreviation: *CCl_4* carbon tetrachloride, *DEN* diethylnitrosamine, *ip* intraperitoneal, *pten* phosphatase and tensin, *Smad 4* SMAD family member

depends on the species-specific microenvironment which is critically different from the tumor developing within the liver [91, 114]. Alternatively, CCA cells may be directly implanted into the bile duct of rodents that enables the study of organotypical interactions between tumor cells and surrounding stroma [115, 116]. In general, it has been shown that orthotopic CCA models are better predictors of drug efficacy and of potentially higher clinical relevance than xenograft models [114]. However, this approach has obvious limitations in the bile duct size of mice being far more time-consuming and technically difficult and therefore more expensive than conventional xenograft models [114].

4.7 Genetically Engineered Mice

4.7.1 p53 Knockout Mice Fed with Carbon Tetrachloride (CCl_4)

Four months administration of CCl_4 in mice harboring a deletion in the p53 gene leads to development of progressive liver injury and fibrosis paralleled by bile duct proliferation [109]. In accordance with the human CCA, p53 gene mutations are frequently observed [117]. Over time, these mice show tumors mimicking human CCA with atypical, infiltrating keratin 19-positive ducts and tubules with a dense collagenous stroma [109]. Similar to the human situation, this mouse model shows CCA development based on a combination of genetic susceptibility with a toxic chronic liver injury [114]. However, this mouse model is not very practicable; since the duration of time needed for tumor development is quite long (29–52 weeks) [114].

4.7.2 Smad4-Pten Knockout Mice

Smad4-Pten knockout mice, harboring a Cre-mediated deletion of phosphatase and tensin homolog (Pten) and SMAD family member 4 (Smad4), a tumor suppressor gene frequently altered in CCA [118] develop tumors histologically similar to intrahepatic CCA [104]. The authors crossbred mice carrying the Smad4 conditional allele (Smad4 Co) and/or the Pten conditional allele (Pten Co) which were crossed with albumin-Cre mice (Alb-Cre). After 2–3 months of age, biliary epithelial hyperplasia is observed, and at 4–7 months, mice show CCAs in all the animals followed by a progressive increase of tumoral intrahepatic nodules. This model is of major relevance for the understanding of the genetic and molecular mechanisms underlying disease development. In accordance with the human situation, PTEN loss has been linked to human CCA development by activation of the pro-proliferative and antiapoptotic PI3K pathway [93, 119]. Although this mouse model enables the investigation of intrahepatic CCA similar to the human situation already at 4–5 months of age, without any necessary further manipulation, limitations of this model include the absence of chronic liver injury and inflammation, the absence of metastases (even in older animals), and the concomitant development of tumors of the salivary glands [93].

4.7.3 The "DEN-Left Median Bile Duct Ligation" Model

Repeated intraperitoneal injection of diethyl-nitrosamine (DEN) in young Balb/c mice, following left median bile duct ligation and oral gavage of DEN, leads to the development of CCA [93, 107]. After 8 weeks, livers showed multifocal cystic hyperplasia of the intrahepatic bile ducts and multifocal cyst formation. At week 12, the biliary epithelium of the hyperplastic foci and the epithelium lining the cysts showed elongated nuclei. After 16 weeks bile duct tumors develop with full development of CCA in these areas at week 28. Despite its complexity requiring technically demanding bile duct surgery and long-term challenge with DEN, it represents the only known non-engineered mouse for CCA development [93].

Over the last few years, a number of HCC and CCA rodent models have been developed, many of them representing valuable tools for investigating some pathophysiological aspects of cancer development. However, due to the complexity of carcinogenesis per se, it is difficult to determine to what extent a single mouse model reproduces the human disease.

Conclusions

Biliary diseases are complex liver diseases in which extra- or intrahepatic bile ducts are affected. Significant research progress has been made the last years to develop animal models that will help understand their pathogenesis. Several limitations however exist which result in no "perfect model" that mirrors human

disease. The use of various aspects of different models might be the way forward to study particular pathogenetic steps in biliary disease.

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