ANIMAL MODELS

A New Mdr2\(^{-/-}\) Mouse Model of Sclerosing Cholangitis with Rapid Fibrosis Progression, Early-Onset Portal Hypertension, and Liver Cancer

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Liver fibrosis, characterized by excessive deposition of extracellular matrix, results from chronic liver injury of different etiologies and represents a major worldwide health problem.\(^1\,2\) Progression of liver fibrosis to cirrhosis leads to complications, including portal hypertension, liver failure, and hepatocellular carcinoma, with high mortality.\(^3\) Primary sclerosing cholangitis (PSC) is an idiopathic, progressive cholestatic liver disease with a male bias and has characteristics of chronic inflammation and fibrosis of the intrahepatic and extrahepatic bile ducts, leading to biliary cirrhosis and cancer.\(^4\) The etiology of PSC is unknown, and no effective therapy exists to halt or reverse progression to cirrhosis outside of liver transplantation. Thus, safe and effective antifibrotic therapies are urgently needed.\(^5\)

Robust and reproducible animal models closely resembling the human disease are critical for target discovery and novel drug development. Mouse models are popular because of their small size, short gestation cycle, and straightforward genetic engineering. For antifibrotic drug development, mouse models are advantageous because they allow for cost-effective preclinical studies and permit thorough target validation using genetic manipulation.\(^6\) However, mice as a species are relatively resistant to the development of progressive liver fibrosis and cirrhosis.\(^7\) In addition, commonly used inbred mouse strains demonstrate varying susceptibility

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to hepatotoxin-induced fibrosis, ranging from the resistant FVB to the susceptible BALB/c strain. This is thought to be associated with at least one susceptibility gene, Hc, and a general bias to profibrotic type 2 helper T-cell immunity.

Mdr2(Abcb4) is a mouse ortholog of human MDR3 (ABCB4) gene encoding for the canalicular phospholipid transporter. Genetic disruption of the Mdr2 gene in mice causes a complete absence of phosphatidylcholine from bile, leading to liver injury, sclerosing cholangitis, and causes a complete absence of phosphatidylcholine from bile, leading to liver injury, sclerosing cholangitis, and cholestasis. We and others have previously reported the Mdr2(Abcb4)/ C0 mouse, on an FVB genetic background (FVB.Mdr2/C0 mouse), as a reproducible model of spontaneously progressive chronic biliary liver disease associated with fibrosis, with histological lesions closely resembling human PSC.

More important, mutations in human ortholog gene MDR3 are associated with a spectrum of liver disorders, including progressive familial intrahepatic cholestasis type 3, low phospholipid–associated cholestasis, parenteral nutrition-induced cholestasis, sepsis-associated cholestasis, and bile duct injury after liver transplantation. Emerging evidence suggests heterozygous MDR3 mutations are frequent in people with cryptogenic biliary cirrhosis. However, compared to severe disease caused by homozygous MDR3 mutations in humans, liver disease in FVB.Mdr2/C0 mice is relatively mild and fibrosis progression is slow.

We hypothesized that slow fibrosis progression in FVB.Mdr2/C0 mouse is due to fibrosis resistance of its FVB genetic background. In this study, we generated and characterized a congenic Mdr2(Abcb4)/C0 mouse on fibrosis-susceptible BALB/c background (BALB/c.Mdr2/C0 mouse) via genetic backcross, aiming to improve the FVB.Mdr2/C0 mouse model for antifibrotic drug testing. We present detailed phenotypic characterization of disease progression in BALB/c.Mdr2/C0 mice in direct comparison with the parental strain for up to 1 year of age.

Materials and Methods

Animal Experimentation

The 5- to 6-week-old wild-type (WT) inbred mouse strains FVB/NJ (number 001800), C57BL/6J (number 000664), and BALB/cJ (number 000651) were purchased from The Jackson Laboratory (Bar Harbor, ME), and BALB/cAnNCrl (number 00282) mice were from Charles River Laboratories (Wilmington, MA). All mice were housed with a 12-hour light-dark cycle and permitted ad libitum consumption of water and a standard chow diet. All animal experiments and procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center, Harvard Medical School (Boston, MA) (protocol number 004-2012).

Table 1 Primers and Probes Used in Real-Time Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Target description (gene)</th>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>β2MG (B2m)</td>
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<td>5’-GACCGGCTCTACTGGGACACATGTTG-3’</td>
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<td></td>
<td>Reverse</td>
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MG, microglobulin; MMP, matrix metalloproteinase; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases.
TAA-Induced Liver Fibrosis Model

Liver fibrosis susceptibility of several inbred mouse strains was analyzed in a repeated liver injury model induced by i.p. thioacetamide (TAA) injections three times per week for 6 weeks, according to the dose-escalating regimen we established previously.21

Generation of Congenic Mdr2 Knockout Mouse on Fibrosis-Susceptible Background

BALB/c.Mdr2<sup>−/−</sup> mice were generated by transferring the Mdr2<sup>−/−</sup> gene knockout from FVB.Mdr2<sup>−/−</sup> mice (FVB.129P2-Acbb4<sup>tm1Bor</sup>; The Jackson Laboratory). Several commonly used inbred strains were analyzed for susceptibility to TAA-induced liver fibrosis, and the most susceptible sub-strain, BALB/cAnNCrl, was selected for conventional backcrossing. Briefly, FVB.Mdr2<sup>−/−</sup> mice were mated to WT BALB/c mice, with heterozygous carriers of Mdr2 mutation in each generation mated to WT BALB/c for 12 generations. The Y chromosome was fixed via breeding female FVB.Mdr2<sup>−/−</sup> carrier to male WT BALB/c in the first breeding cycle; male Mdr2 mutation carriers were bred to WT BALB/c females for the subsequent 11 generations. Mutation carriers in N12 progeny were intercrossed to obtain mice homozygous for Mdr2 mutation and establish the BALB/c.Mdr2<sup>−/−</sup>/C<sup>0</sup> colony. Liver tumors in 7- and 12-month-old mice were evaluated macroscopically and microscopically.

Hepatic Collagen Content Determination

Hepatic collagen content was determined as relative hydroxyproline (μg per 100 mg of wet liver) in 300- to 400-mg liver samples from two different lobes (representing >10% of whole liver) after hydrolysis in 6N HCl for 16 hours at 110°C, as described.22 Total hydroxyproline (mg/whole liver) was calculated on the basis of individual liver weights and the corresponding relative hydroxyproline content.22

Fibrotic Matrix Stability Assessment

Extracellular membrane stability was assessed biochemically ex vivo by complete fibrotic matrix fractionation via serial extractions, and solubilized collagen quantification in each fraction was performed as previously described,21 with modifications. Briefly, 500 mg of snap-frozen tissue from two liver lobes was homogenized, and a series of overnight extractions (dilution 1:20, w/v) in increasingly harsh conditions was performed to obtain the following collagen fractions: acetic acid–soluble fraction (soluble, freshly secreted collagens and mature collagens), pepsin-soluble fraction (fibrillar and moderately cross-linked collagens), and insoluble, highly cross-linked collagens. Collagen content solubilized in each step was quantified biochemically via hydroxyproline after complete acidic hydrolysis and expressed as percentage of hydroxyproline recovered in all fractions.

IHC and Immunoblotting

Immunohistochemical (IHC), hematoxylin and eosin, connective tissue (Sirius Red), and reticulin stains were performed in formalin-fixed, paraffin-embedded liver sections of snap-frozen liver pieces, as described previously.22–24 Anti–α-smooth muscle actin (α-SMA; ab5694; dilution 1:400; Abcam, Cambridge, MA) and anti–pan-cytokeratin (Z0622; dilution 1:50; Dako, Glostrup, Denmark) antibodies were used as primary antibodies for IHC. Images were captured using Zeiss Axioimager M1 (Carl Zeiss, Oberkochen, Germany). For Western blot analysis, 15 μg of tissue lysate per lane was probed with anti–α-SMA antibody (M0851; dilution 1:1000; Dako) or anti-β actin (number 4970; dilution 1:2000; Cell Signaling Technology, Danvers, MA), as described.22

Real-Time Quantitative RT-PCR

Relative mRNA levels were quantified in total liver RNA by real-time quantitative RT-PCR on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using the TaqMan method, as described in detail.17,21,23 Sequences of primers and probes used in this study are summarized in Table 1.

Figure 1  Analysis of fibrotic responses of common inbred mouse strains to repeated thioacetamide (TAA)—induced liver injury identifies BALB/c substrain as highly fibrosus susceptible. A: Representative low-magnification images of connective tissue stain (Sirius Red) from livers of FVB/NJ, C57BL/6J, BALB/cJ, and BALB/cAnNCrl mice treated with hepatotoxin TAA for 6 weeks. B: Relative and total hepatic collagen content. Data are means ± SEM. n = 8 to 9 per group for TAA; n = 5 for controls. **P < 0.001, one-way analysis of variance, followed by Bonferroni post test. Original magnification, ×50 (A).
Portal Venous Pressure Measurement

Mice were anesthetized with isoflurane (1.5% v/v) via precise vaporizer. After laparotomy, the portal vein was cannulated through an iliocolic vein, and portal pressure was measured directly by inserting a 1.2-Fr high-fidelity pressure catheter (Scisense, London, ON, Canada). Pressure signals were recorded at 2 kHz for 5 minutes, and analyzed using PowerLab software chart 5.5.6 (ADInstruments, Colorado Springs, CO).

Serum Chemistry

Serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase (ALP), and total bilirubin were measured using an automated Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME), according to the manufacturer’s recommendations.

Statistical Analysis

Data are expressed as means ± SEM, and statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA) and Graph-Pad Prism 5 (GraphPad Software, San Diego, CA). Multiple comparisons were performed by two-way analysis of variance, followed by Bonferroni post test, and comparisons between two groups were performed by the Student’s t-test. Differences between experimental groups with \( P < 0.05 \) were considered significant.

Results

Identification of Highly Fibrosis-Susceptible BALB/c Substrain in Mice

We evaluated susceptibility to liver fibrosis induced by repeated TAA injections for 6 weeks in four commonly used...
strains of inbred mice [FVB/NJ, C57BL/6J, and two BALB/c substrains (BALB/cJ and BALB/cAnNCrl)]. Connective tissue staining revealed significant differences in fibrosis degree (FVB/NJ < C57BL/6J < BALB/cJ < BALB/cAnNCrl), ranging from absence of significant fibrosis in FVB/NJ mice to advanced, bridging fibrosis in BALB/cAnNCrl (Figure 1A). Liver collagen content, as determined biochemically via hydroxyproline, increased significantly in all strains, but to a dramatically different extent: relative hydroxyproline (per 100 mg liver) increased by 24% (FVB/NJ), 85% (C57BL/6J), 143% (BALB/cJ), and 205% (BALB/cAnNCrl) compared to their healthy strain-matched controls. Differences were even more significant for total collagen deposition (per whole liver), which was 1.4-fold in FVB/NJ, 1.95-fold in C57BL/6J, 2.52-fold in BALB/cJ, and 3.67-fold in BALB/cAnNCrl (Figure 1B and Supplemental Table S1). Interestingly, BALB/c substrains demonstrated considerable differences in fibrosis, with almost twofold greater absolute amount of collagen deposited in BALB/cAnNCrl compared to BALB/cJ. Splenomegaly developed significantly higher at 8 weeks and thick septae formation by 12 weeks (Figure 2A). Quantitatively, collagen content in the liver of male BALB/cJ mice is cross-linked to a greater extent than that of FVB.Mdr2/C0/C0 mice, 1.435 mg compared to 0.535 mg of hydroxyproline in FVB.Mdr2/C0/C0 mice (Figure 2C and Supplemental Table S2). Net collagen deposition from week 4 through 12, calculated from an absolute increase in total hydroxyproline content (per whole liver), was also approximately threefold greater in BALB/c.Mdr2/C0/C0 mice, 1.435 mg compared to 0.535 mg of hydroxyproline in FVB.Mdr2/C0/C0 mice (Figure 2C and Supplemental Table S3). We evaluated fibrotic matrix stability in both strains at 8 weeks of age to assess the extent of collagen cross-linking using stepwise collagen extractions.21 In FVB.Mdr2/C0/C0 mice, highly cross-linked (insoluble) collagen fraction represented 19.9% ± 1.4% of total, a 73% increase compared to age-matched WT FVB mice (11.5% ± 0.6%) (P < 0.01). In BALB/c.Mdr2/C0/C0 mice, insoluble collagens represented 42.2% ± 1.8% fraction in BALB/c.Mdr2/C0/C0 mice, a 210% increase compared to healthy WT controls (13.6% ± 0.8%) (P < 0.001) (Figure 2D). This suggests that fibrotic matrix in BALB/c.Mdr2/C0/C0 mice is cross-linked to a greater degree than in FVB.Mdr2/C0/C0 mice. Rapid fibrosis progression in BALB/c.Mdr2/C0/C0 mice cannot be explained by...

**Generation of BALB/c.Mdr2/C0/C0 Mouse**

Congenic Mdr2/C0/C0 mouse on BALB/cAnNCrl genetic background (BALB/c.Mdr2/C0/C0 from here forward) was generated using conventional backcross onto BALB/cAnNCrl from parental FVB.129P2-Abcb4m1Bor (FVB.Mdr2/C0/C0 from here forward) (The Jackson Laboratory) for 12 generations, as described in detail in **Materials and Methods**. Homozygous BALB/c.Mdr2/C0/C0 mice were viable and fertile, with no gross abnormalities or significant mortality compared to parental FVB.Mdr2/C0/C0 strain when observed up to 1 year of age. Homozygous mating of both FVB.Mdr2/C0/C0 and BALB/c.Mdr2/C0/C0 was used to generate mice for phenotype analysis.

**BALB/c.Mdr2/C0/C0 Mice Demonstrate Dramatic Acceleration of Liver Fibrosis Progression**

We performed direct phenotypic comparison of liver fibrosis development in novel BALB/c.Mdr2/C0/C0 mice and parental FVB.Mdr2/C0/C0 mice at early time points of 4, 8, and 12 weeks of age. Both strains spontaneously developed periductal onion-skin type fibrotic lesions starting from 4 weeks of age (Figure 2, A and B). When compared to FVB.Mdr2/C0/C0 mice, fibrosis in BALB/c.Mdr2/C0/C0 mice was dramatically accelerated, with histological signs of bridging fibrosis already at 8 weeks and thick septae formation by 12 weeks (Figure 2A). Quantitatively, collagen content in the liver of male BALB/c.Mdr2/C0/C0 mice was significantly higher at any time point than that of FVB.Mdr2/C0/C0 mice: by 2.2-fold at week 4, 3.1-fold at week 8, and 3.3-fold at week 12

![Figure 3](https://ajp.amjpathol.org/article/S0002-9092(17)32358-9/fig-3)

**Figure 3** Genetic background profoundly alters fibrosis-related gene expression profile in Mdr2/C0/C0 mice. A: Pro-fibrotic hepatic gene expression in Mdr2/C0/C0 mice at 4, 8, and 12 weeks demonstrates notable overexpression of procollagen α1(I), tissue inhibitor of metalloproteinase 1 (Timp-1), and transforming growth factor (Tgf)-β in BALB/c.Mdr2/C0/C0 mice compared to parental FVB strain levels. B: Matrix metalloproteinase (MMP) hepatic expression in FVB.Mdr2/C0/C0 and BALB/c.Mdr2/C0/C0 mice reveals significant differences in pro-fibrotic collagenase Mmp-13 expression. Data are expressed as means ± SEM, as fold increase to respective age-matched WT controls. n = 3 to 9 per group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared to FVB.Mdr2/C0/C0 mice, two-way analysis of variance, followed by Bonferroni post test.
fibrosis in BALB/c.Mdr2⁻/⁻ mice was associated with robust increases in profibrogenic transcripts, such as procollagen α1(I) and (cholangiocyte-derived) transforming growth factor (Tgf)-β2, twofold to fivefold above parental strain levels at ages of 8 and 12 weeks (Figure 3A). This did not occur with other profibrogenic genes, such as TGF-β1, which was only transiently elevated at 4 weeks, and integrin β6, which was overexpressed at similar levels in both strains (Figure 3A). Interestingly, profibrolytic Mmp-13 expression was suppressed in BALB/c.Mdr2⁻/⁻ mice at 8 and 12 weeks compared to parental strain, whereas Mmp-3 levels showed no significant differences (Figure 3B). On the other hand, endogenous MMP inhibitor, tissue inhibitor of metalloproteinases 1, was up-regulated up to fivefold at every time point in BALB/c.Mdr2⁻/⁻ mice compared to FVB.Mdr2⁻/⁻ mice (Figure 3A).

Fibrogenic Cell Activation Is Amplified in BALB/c.Mdr2⁻/⁻ Mice

Biliary liver fibrosis is associated with proliferation of cholangiocytes and adult hepatic progenitors (ductular reaction) greater inflammation or liver injury, because both strains demonstrated comparable increases in serum alanine aminotransferase and aspartate aminotransferase (Supplemental Figure S1). Interestingly, although FVB.Mdr2⁻/⁻ mice demonstrated more severe fibrosis in females, as expected, significant male bias was found in BALB/c.Mdr2⁻/⁻ mice, with significantly higher hepatic collagen content in males starting at 8 weeks of age (P < 0.01) (Supplemental Table S2).

Profiling of the Fibrosis-Related Gene Expression Reveals Alteration in Both Profibrogenic and Profibrolytic Pathways in BALB/c.Mdr2⁻/⁻ Mice

To investigate the relative contribution of increased fibrogenesis versus inhibited fibrolysis in the rapid fibrosis progression of BALB/c.Mdr2⁻/⁻ mice, the hepatic expression levels of several profibrogenic genes and putatively profibrolytic matrix metalloproteinases (MMP) in males of both strains were analyzed by real-time quantitative RT-PCR. Aggressive
and activation of hepatic stellate cells (HSCs)/portal myofibroblasts (MFs). We assessed ductular reaction and activation of HSC/MF by IHC for progenitors/cholangiocyte marker, pan-cytokeratin, and HSC/MF activation marker, α-SMA. Both BALB/c.Mdr2−/− and FVB.Mdr2−/− mice demonstrated pronounced ductular reaction in periportal space, often extending into parenchyma, without notable difference on IHC staining (Figure 4A). However, serum ALP levels were higher in BALB/c.Mdr2−/− mice and increased with age, whereas they decreased in FVB.Mdr2−/− mice by 12 weeks (P < 0.001) (Supplemental Figure S1B). α-SMA-positive MFs were found mostly in peribiliary fibrotic bands, but were notably more abundant in BALB/c.Mdr2−/− mice when compared to the parental strain. Western blot analysis confirmed quantitative strain differences in α-SMA expression, which was severalfold higher in BALB/c.Mdr2−/− mice (P < 0.001) (Figure 4B).

Portal hypertension is the most frequent complication of liver fibrosis, leading to fatal complications, such as variceal bleeding. We evaluated portal pressure in both Mdr2−/− strains from 4 to 12 weeks of age by direct invasive measurement. Both strains of Mdr2−/− mice demonstrate increased portal pressure compared to respective healthy WT controls (FVB/NJ, 4.3 ± 0.1 mmHg; BALB/cAnNCrl,}

![Image](image-url)
4.9 ± 0.2 mmHg); however, significant differences between \( \textit{Mdr2}\textsuperscript{+/−} \) strains were found at every time point starting from 4 weeks of age (Figure 5A). Portal pressure in BALB/c/\( \textit{Mdr2}\textsuperscript{+/−} \) mice increased at a significantly higher pace compared to parental FVB/\( \textit{Mdr2}\textsuperscript{+/−} \) mice (\( P < 0.001 \)) (Figure 5A), peaking at 12 mmHg at 12 weeks (compared to 8.2 mmHg in FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice), and was accompanied by severe splenomegaly (\( P < 0.001 \)) (Supplemental Table S3). Interestingly, careful histological examination at high magnification revealed prominent sinusoidal fibrosis in BALB/c/\( \textit{Mdr2}\textsuperscript{+/−} \) mice starting at 8 weeks, but not in age-matched FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice (Figure 5B), which can possibly be responsible for early-onset, severe portal hypertension in BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice.

Development of Cirrhosis and Primary Liver Cancer Is Accelerated in Aged BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) Mice

Finally, we evaluated the long-term liver disease outcomes in BALB/c/\( \textit{Mdr2}\textsuperscript{+/−} \) and FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice at 7 and 12 months of age. Compared to the parental strain, BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice lost weight at 12 months (\( P < 0.001 \): 21.5% versus 2.9% reduction relative to respective healthy WT controls, respectively) (Supplemental Table S3) and demonstrated histological signs of cirrhosis and massive collagen deposition in the liver (Figure 6, A and B). Notably, total bilirubin in serum of aged BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice was severalfold higher at 7 months compared to FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice and continued to increase through 12 months of age, indicating a progressive loss of liver function (Figure 6C). Biliary injury marker ALP was also elevated in BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice compared to FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice at 7 and 12 months of age (Figure 6C). Macroscopic examination of livers revealed that BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice developed visible liver tumors (>1 mm) starting at 7 months of age (Figure 7A and Supplemental Table S4), whereas tumors in FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice were only detected at 12 months. The overall tumor burden was also significantly higher in BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice compared to parental strain (\( P < 0.01 \)) (Figure 7B). Liver tumors in both strains were examined histologically by an experienced pathologist (I.N.) and were classified as hepatocellular carcinoma, on the basis of morphological characteristics and typical pattern of absent reticulin staining (Figure 7C).

Discussion

Herein, we generated and characterized a novel congenic \( \textit{Mdr2}\textsuperscript{−/−} \) mouse on a fibrosis-susceptible background. Direct comparison of BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice with parental strain (fibrosis-resistant FVB/NJ) demonstrates that genetic background potently influences the resultant phenotype, with dramatic acceleration of liver fibrosis, early-onset portal hypertension, and primary liver cancer in BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice.

Inbred mice are commonly used as experimental small animal models of liver fibrosis. However, mice as a species are relatively resistant to liver fibrosis compared to, for example, rats, and achieving progressive fibrosis, and especially cirrhosis, in mouse models is challenging.\(^7\) This can be overcome by careful selection of inbred strains and fine-tuning of fibrosis induction protocols. Recently, we successfully optimized TAA- and CCl\(_4\)-induced models to achieve robust progressive fibrosis in resistant C57Bl/6 strain, through more frequent administration of escalating doses of hepatotoxins.\(^{28}\) Hillebrandt et al\(^3\) reported remarkable differences in seven inbred mouse strains in CCl\(_4\)-induced liver fibrosis, with BALB/cj being most susceptible. We confirm such strain differences in a mechanistically different (TAA-induced) fibrosis model and extend characterization to additional BALB/cAnNCrl substrain, in which fibrosis was strikingly greater than BALB/cj (Figure 1).

Phenotypic analysis of young \( \textit{Mdr2}\textsuperscript{−/−} \) mice backcrossed onto BALB/cAnNCrl background showed an approximately threefold increase in resultant liver fibrosis compared to parental FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) (Figure 2C). Quantitatively, extensive fibrosis in BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice exceeded that observed in the same strain in a TAA model (Figure 1B). Rate of fibrosis progression from 4 to 12 weeks of age was high, as estimated by absolute increase in total hepatic collagen content, with an average 179 \( \mu \)g per liver per week of hydroxyproline deposited in male BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice compared to 67 \( \mu \)g per liver per week in age- and sex-matched FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) mice (Supplemental Table S2). For comparison, the most aggressive liver fibrosis model used in our laboratory, to date, a 12-week escalating dose of chronic CCl\(_4\) in C57Bl6 mice, demonstrates a progression rate of 77 \( \mu \)g of hydroxyproline per liver per week.\(^{21}\) We and others have shown that collagen cross-linking is prominent in progressive fibrosis models, and possibly contributes to fibrosis progression and limits spontaneous reversal in fibrosis models.\(^{21,29}\) Direct biochemical analysis of fibrotic matrix in our BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice revealed that >40% of collagen is highly cross-linked (Figure 2D), compared to 20% in parental FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) and 27% in the CCl\(_4\)-induced model.\(^{31}\) Future studies should determine to what extent increased collagen cross-linking contributes to the susceptibility to fibrosis of BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mice.

Previously, the Lammert laboratory reported generation of \( \textit{Mdr2}\textsuperscript{−/−} \) mice on a background of a different BALB/c substrain (BALB/cJ),\(^{30}\) which was subsequently characterized longitudinally from 4 to 28 weeks.\(^{31,32}\) Although direct phenotypic comparison to parental FVB/\( \textit{Mdr2}\textsuperscript{−/−} \) strain was not performed, objective fibrosis parameters indicate that BALB/cj/\( \textit{Mdr2}\textsuperscript{−/−} \) strain, in terms of fibrotic response, is an improvement over fibrosis-resistant FVB, but develops less fibrosis than our new BALB/c/\( \textit{Mdr2}\textsuperscript{−/−} \) mouse. The reported gold standard quantitative assessment of fibrosis...
via hepatic hydroxyproline content is 48.0 ± 7.6 μg/100 mg in BALB/c.Mdr2−/− mice at 8 weeks of age,21 compared to 70.3 ± 3.8 μg/100 mg in BALB/c.Mdr2−/− mice of the same age (Figure 2C and Supplemental Table S2). This is consistent with quantitative differences in fibrotic phenotype we found between substrains BALB/c and BALB/cAnNCrl in response to chronic TAA (Figure 1). Such significant variations of disease phenotype between BALB/c substrains, however surprising, were previously reported in other disease models.33,34 Our data suggest the substrain selection is important in modeling liver fibrosis, and provide a rationale for further studies to identify specific susceptibility gene(s) in BALB/cAnNCrl mice.

Mdr2−/− mice develop chronic liver injury closely resembling histological features of human PSC, such as cholangitis, periductular fibrosis (onion skinning), and bile duct proliferation.15–17 In addition to these typical findings, as well as hepatosplenomegaly observed in both Mdr2−/− strains, aged BALB/c.Mdr2−/− mice demonstrated weight loss, hyperbilirubinemia, and higher levels of ALP (Figure 6A and Supplemental Table S3), which are other important aspects of human PSC.4 More important, male BALB/c.Mdr2−/− mice develop more severe fibrosis compared to females, in contrast to parental FVB.Mdr2−/− mice, in which males are less fibrotic25 (Supplemental Table S2). However, we have not been able to detect instances of cholangiocarcinoma, frequent in human PSC. Although primary liver cancer develops much earlier in BALB/c.Mdr2−/− mice, all tumors in both strains were classified as hepatocellular carcinoma by an experienced pathologist (L.N.).

Considering comparable serum alanine aminotransferase levels in both Mdr2−/− strains, dramatic differences in fibrosis progression are not simply due to more liver injury in BALB/c.Mdr2−/− mice. Marked strain differences in α-SMA expression, an activation marker of MF cells, and periductular accumulation of α-SMA+ cells in Mdr2−/− mice on BALB/cAnNCrl background point to central role of HSC/MF activation in our model (Figure 4). Furthermore, gene expression profiling data indicate profound strain differences in both profibrogenic and profibrolytic pathways that are consistent with amplified fibrogenic activation of MFs in BALB/c.Mdr2−/− mice. Taken together, these data show that fibrosis acceleration in BALB/c.Mdr2−/− mice is associated with increased activation of HSC/MF, and results from both increased fibrogenesis and suppressed fibrolysis, as revealed by remarkable elevation in procollagen transcription (Cola1 mRNA), on one hand, and down-regulation of profibrolytic collagenase (Mmp-13) and concomitant overexpression of its inhibitor, tissue inhibitor of metalloproteinase 1, on the other hand (Figure 5).

Portal hypertension is one of the most important pathophysiological consequences of liver fibrosis. A hepatic venous pressure gradient >10 mmHg predicts cirrhosis decompensation35,36 and >12 mmHg predicts variceal bleeding.37–39 We invasively measured portal pressure in both Mdr2−/− strains, which so far has not been assessed in this model. BALB/c.Mdr2−/− mice demonstrated a significant increase in portal pressure from the earliest 4-week time point (compared to WT controls and FVB.Mdr2−/− mice), elevated up to threefold when compared to healthy WT mice. Given that in normal conditions human hepatic venous pressure gradient is <5 mmHg,35 which is equivalent to the portal pressure in WT BALB/cAnNCrl mice, portal pressure >10 mmHg in BALB/c.Mdr2−/− mice as early as 8 weeks of age can be considered clinically significant, and can be used as an end point in formal antifibrotic drug testing.

Host genetic factors are thought to play a key role in the variability in hepatic fibrosis progression in individual patients.40 Our new model offers a unique opportunity to discover new biomarkers and test diagnostic approaches aiming to assess the activity of fibrotic process and/or identify individuals at risk of progression into cirrhosis. Such tests can identify patients in greatest need of antifibrotic therapies, and help stratify patients in clinical trials to reduce the overall number of patients required and overcome prohibitive costs.31 Because both strains develop hepatic fibrosis due to the exact same mutation, with differences in fibrosis determined solely by genetic makeup, we assert that our model recapitulates significant differences in the rate of fibrosis progression in humans with chronic liver disease.

In conclusion, we generated a novel BALB/c.Mdr2−/− mouse that spontaneously develops PSC-like lesions and is characterized by unprecedented degree and rapidity of hepatic fibrosis progression and clinically relevant complications of cirrhosis. This new rapid fibroser mouse represents a major improvement over existing murine liver fibrosis models, and may facilitate the development of antifibrotic and anti-cancer drugs, biomarker discovery, and basic studies into the mechanisms of biliary fibrosis progression.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.10.013.

References

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