ANIMAL MODELS

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

Naoki Ikenaga,^{*} Susan B. Liu,^{*} Deanna Y. Sverdlov,^{*} Shuhei Yoshida,^{*} Imad Nasser,[†] Qingen Ke,[‡] Peter M. Kang,[‡] and Yury Popov^{*}

From the Division of Gastroenterology and Hepatology,^{*} Department of Medicine, the Department of Pathology,[†] and the Division of Cardiology,[‡] Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Accepted for publication
October 9, 2014.

Address correspondence to
Yury Popov, M.D., Ph.D.,
Division of Gastroenterology
and Hepatology, Department of
Medicine, Beth Israel
Deaconess Medical Center,
Harvard Medical School, 330
Brookline Ave, Boston,
MA 02115. E-mail: ypopov@bidmc.harvard.edu.

We previously characterized the *Mdr2*(*Abcb4*)^{-/-} mouse as a reproducible model of chronic biliary liver disease. However, it demonstrates relatively slow fibrosis progression, possibly due to its fibrosis-resistant genetic background. We aimed to improve the model by moving it onto a fibrosis-susceptible background. We generated novel BALB/c.*Mdr2*^{-/-} mouse via genetic backcross onto highly fibrosis-susceptible BALB/c substrain, identified in inbred mouse strain screening. Liver fibrosis, portal pressure, and hepatic tumor burden in BALB/c.*Mdr2*^{-/-} mice were studied up to 1 year of age in direct comparison to parental strain FVB.*Mdr2*^{-/-}. BALB/c.*Mdr2*^{-/-} mice developed periductular onion-skin type fibrotic lesions and pronounced ductular reaction starting from 4 weeks of age. Compared to parental strain, BALB/c.*Mdr2*^{-/-} mice demonstrated dramatically accelerated liver fibrosis, with threefold increase in collagen deposition and bridging fibrosis/early signs of cirrhosis at 12 weeks. This was accompanied by early-onset severe portal hypertension and twofold to fourfold increase in profibrogenic transcripts *Col1a1* [procollagen α 1(I)], *Tgfb1*, and *Timp1*. Primary liver cancers in BALB/c.*Mdr2*^{-/-} developed earlier, with greater tumor burden compared to FVB.*Mdr2*^{-/-}. BALB/c.*Mdr2*^{-/-} mice have unprecedented degree and rapidity of hepatic fibrosis progression and clinically relevant cirrhosis complications, such as early-onset portal hypertension and primary liver cancers. This new model will facilitate development of antifibrotic drugs and studies into mechanisms of biliary fibrosis progression. (*Am J Pathol* 2015, 185: 325–334; <http://dx.doi.org/10.1016/j.ajpath.2014.10.013>)

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

Supported by an institutional grant from the Department of Medicine, Beth Israel Deaconess Medical Center (Y.P.).

Disclosures: None declared.

to hepatotoxin-induced fibrosis, ranging from the resistant FVB to the susceptible BALB/c strain.⁸ This is thought to be associated with at least one susceptibility gene, Hc,⁹ and a general bias to profibrotic type 2 helper T-cell immunity.¹⁰

Mdr2(Abcb4) is a mouse ortholog of human *MDR3* (*ABCB4*) gene encoding for the canalicular phospholipid transporter.¹¹ Genetic disruption of the *Mdr2* gene in mice causes a complete absence of phosphatidylcholine from bile,¹² leading to liver injury, sclerosing cholangitis,¹³ and cholelithiasis.¹⁴ We and others have previously reported the *Mdr2(Abcb4)*^{-/-} mouse, on an FVB genetic background (FVB.*Mdr2*^{-/-} mouse), as a reproducible model of spontaneously progressive chronic biliary liver disease associated with fibrosis, with histological lesions closely resembling human PSC.¹⁵⁻¹⁷ 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 cholelithiasis, parenteral nutrition-induced cholestasis, sepsis-associated cholestasis, and bile duct injury after liver transplantation.^{11,18} Emerging evidence suggests heterozygous *MDR3* mutations are frequent in people with cryptogenic biliary cirrhosis.¹⁹ However, compared to severe disease caused by homozygous *MDR3* mutations in humans,¹⁸ liver disease in FVB.*Mdr2*^{-/-} mice is relatively mild and fibrosis progression is slow.²⁰

We hypothesized that slow fibrosis progression in FVB.*Mdr2*^{-/-} mouse is due to fibrosis resistance of its FVB genetic background. In this study, we generated and characterized a congenic *Mdr2(Abcb4)*^{-/-} mouse on fibrosis-susceptible BALB/c background (BALB/c.*Mdr2*^{-/-} mouse) via genetic backcross, aiming to improve the FVB.*Mdr2*^{-/-} mouse model for antifibrotic drug testing. We present detailed phenotypic characterization of disease progression in BALB/c.*Mdr2*^{-/-} 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 028) 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

Target description (gene)	Primer	Sequence
β2MG (<i>B2m</i>)	Forward	5'-CTGATACATACGCCTGCAGAGTTAA-3'
	Probe	5'-GACCGTCTACTGGGATCGAGACATGTG-3'
	Reverse	5'-ATGAATCTTCAGAGCATCATGAT-3'
Procollagen α1(I) (<i>Col1a1</i>)	Forward	5'-TCCGGCTCCTGCTCCTCTTA-3'
	Probe	5'-TTCTTGCCATGCGTCAGGAGGG-3'
	Reverse	5'-GTATGCAGCTGACTTCAGGGATGT-3'
TGF-β1 (<i>Tgfb1</i>)	Forward	5'-AGAGGTCACCCGCGTGCTAA-3'
	Probe	5'-ACCGCAACAACGCCATCTATGAGAAAACCA-3'
	Reverse	5'-TCCCGAATGTCTGACGTATTGA-3'
TGF-β2 (<i>Tgfb2</i>)	Forward	5'-GTCCAGCCGGCGGAA-3'
	Probe	5'-CGCTTTGGATGCTGCCTACTGCTTTAGAAAT-3'
	Reverse	5'-GCGAAGGCAGCAATTATCCT-3'
Integrin β6 (<i>Itgb6</i>)	Forward	5'-GCAGAACGCTCTAAGGCCAA-3'
	Probe	5'-TGGCAAACGGGAACCAATCCTCTGT-3'
	Reverse	5'-AAAGTGCTGGTGAACCTCG-3'
Mmp-2 (<i>Mmp2</i>)	Forward	5'-CCGAGGACTATGACCGGGATAA-3'
	Probe	5'-TCTGCCCCGAGACCGCTATGTCCA-3'
	Reverse	5'-CTTGTTGCCAGGAAAGTGAAG-3'
Mmp-3 (<i>Mmp3</i>)	Forward	5'-GATGAACGATGGACAGAGGATG-3'
	Probe	5'-TGGTACCAACCTATTCCTGGTTGCTGC-3'
	Reverse	5'-AGGGAGTGGCCAAGTTCATG-3'
Mmp-13 (<i>Mmp13</i>)	Forward	5'-GGAAGACCTCTTCTTCTCT-3'
	Probe	5'-TCTGGTTAATCATCATCAACTCCACACGT-3'
	Reverse	5'-TCATAGACAGCATCTACTTTGTT-3'
Timp-1 (<i>Timp1</i>)	Forward	5'-TCCTCTTGTTGCTATCACTGATAGCTT-3'
	Probe	5'-TTCTGCAACTCGGACCTGGTCATAAGG-3'
	Reverse	5'-CGCTGGTATAAGGTGGTCTCGTT-3'

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*^{-/-} mice were generated by transferring the *Mdr2* gene knockout from FVB.*Mdr2*^{-/-} mice (FVB.129P2-Abcb4^{tm1Bor}; The Jackson Laboratory). Several commonly used inbred strains were analyzed for susceptibility to TAA-induced liver fibrosis, and the most susceptible substrain, BALB/cAnNCrI, was selected for conventional backcrossing. Briefly, FVB.*Mdr2*^{-/-} 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*^{-/-} 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*^{-/-} 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.¹⁷ 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 fibrosis susceptible. **A:** Representative low-magnification images of connective tissue stain (Sirius Red) from livers of FVB/NJ, C57BL/6J, BALB/cJ, and BALB/cAnNCrI mice treated with hepatotoxin TAA for 6 weeks. **B:** Relative and total hepatic collagen content. Data are means $\pm$ 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, $\times 50$ (**A**).

Figure 2 Novel BALB/c.*Mdr2*^{-/-} mice demonstrate dramatic acceleration of liver fibrosis progression compared to parental FVB.*Mdr2*^{-/-} mice. **A:** Representative low-magnification images of connective tissue stain (Sirius Red) from livers of male FVB.*Mdr2*^{-/-} and BALB/c.*Mdr2*^{-/-} mice at age 4 to 12 weeks. **B:** Typical portal primary sclerosing cholangitis-like lesions demonstrate enlarged bile ducts embedded in onion skin fibrotic bands. The areas of the insets in A are shown, respectively. **C:** Relative and total hepatic collagen content in *Mdr2*^{-/-} mice. **D:** Fibrotic matrix is highly cross-linked in BALB/c.*Mdr2*^{-/-} mice, as determined by serial collagen extraction/fractionation assay. Data are expressed as means $\pm$ SEM (C). $n = 8$ to 15 per group (C); $n = 8$, 8-week-old *Mdr2*^{-/-} mice; $n = 4$, WT mice (D). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to FVB.*Mdr2*^{-/-} mice, two-way analysis of variance, Bonferroni post test (C); *** $P < 0.001$ for highly cross-linked (insoluble) collagen fraction, Student's *t*-test (D). Original magnifications: $\times 50$ (A); $\times 200$ (B, insets from 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 ilioocolic 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 $\pm$ 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/cAnNCr)]. Connective tissue staining revealed significant differences in fibrosis degree (FVB/NJ < C57BL/6J < BALB/cJ < BALB/cAnNCr), ranging from absence of significant fibrosis in FVB/NJ mice to advanced, bridging fibrosis in BALB/cAnNCr (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/cAnNCr) 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/cAnNCr (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/cAnNCr compared to BALB/cJ. Splenomegaly developed only in BALB/cAnNCr mice among all strains tested (Supplemental Table S1). On the basis of these results, BALB/cAnNCr substrain was selected for *Mdr2*^{-/-} mutation transfer from the fibrosis-resistant FVB background.

Generation of BALB/c.*Mdr2*^{-/-} Mouse

Congenic *Mdr2*^{-/-} mouse on BALB/cAnNCr genetic background (BALB/c.*Mdr2*^{-/-} from here forward) was generated using conventional backcross onto BALB/cAnNCr from parental FVB.129P2-Abcb4^{tm1Bor} (FVB.*Mdr2*^{-/-} from here forward) (The Jackson Laboratory) for 12 generations, as described in detail in *Materials and Methods*. Homozygous BALB/c.*Mdr2*^{-/-} mice were viable and fertile, with no gross abnormalities or significant mortality compared to parental FVB.*Mdr2*^{-/-} strain when observed up to 1 year of age. Homozygous mating of both FVB.*Mdr2*^{-/-} and BALB/c.*Mdr2*^{-/-} was used to generate mice for phenotypic analysis.

BALB/c.*Mdr2*^{-/-} Mice Demonstrate Dramatic Acceleration of Liver Fibrosis Progression

We performed direct phenotypic comparison of liver fibrosis development in novel BALB/c.*Mdr2*^{-/-} mice and parental FVB.*Mdr2*^{-/-} 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*^{-/-} mice, fibrosis in BALB/c.*Mdr2*^{-/-} 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*^{-/-} mice was significantly higher at any time point than that of FVB.*Mdr2*^{-/-} mice: by 2.2-fold at week 4, 3.1-fold at week 8, and 3.3-fold at week 12

(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*^{-/-} mice, 1.435 mg compared to 0.535 mg of hydroxyproline in FVB.*Mdr2*^{-/-} 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*^{-/-} 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*^{-/-} mice, insoluble collagens represented 42.2% ± 1.8% fraction in BALB/c.*Mdr2*^{-/-} mice, a 210% increase compared to healthy WT controls (13.6% ± 0.8%) (*P* < 0.001), which was significantly greater than in parental strain (*P* < 0.001) (Figure 2D). This suggests that fibrotic matrix in BALB/c.*Mdr2*^{-/-} mice is cross-linked to a greater degree than in FVB.*Mdr2*^{-/-} mice. Rapid fibrosis progression in BALB/c.*Mdr2*^{-/-} mice cannot be explained by

Figure 3 Genetic background profoundly alters fibrosis-related gene expression profile in *Mdr2*^{-/-} mice. **A:** Profibrogenic hepatic gene expression in *Mdr2*^{-/-} 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*^{-/-} mice above parental FVB strain levels. **B:** Matrix metalloproteinase (MMP) hepatic expression in FVB.*Mdr2*^{-/-} and BALB/c.*Mdr2*^{-/-} mice reveals significant differences in profibrotic 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*^{-/-} mice, two-way analysis of variance, followed by Bonferroni post test.

Figure 4 Fibrogenic cell activation increases in BALB/c.*Mdr2*^{-/-} mice. **A:** Serial liver sections stained with ductular cell marker pan-cytokeratin (p-CK) and α-smooth muscle actin (SMA). Activated α-SMA⁺ myofibroblasts within periductular fibrotic band are greatly increased in BALB/c.*Mdr2*^{-/-} mice. Representative images from 8-week-old *Mdr2*^{-/-} mice. **B:** Western blot and densitometry analyses of α-SMA protein expression in liver lysates of 8-week-old *Mdr2*^{-/-} mice. ****P* < 0.001 compared to FVB.*Mdr2*^{-/-} mice (Student's *t*-test). Original magnification, ×200 (**A**).

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,²⁵ 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

fibrosis in BALB/c.*Mdr2*^{-/-} mice was associated with robust increases in profibrogenic transcripts, such as procollagen α1(I) and (cholangiocyte-derived²⁶) transforming growth factor (Tgf)-β2, twofold to fivefold above parental strain levels^{17,23} 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)

Figure 5 BALB/c.*Mdr2*^{-/-} mice demonstrate early-onset severe portal hypertension. **A:** Invasive measurement of portal venous pressure was performed in wild-type (WT) and *Mdr2*^{-/-} mice of up to 12 weeks of age (left). Representative records of the portal vein pressure in 8-week-old FVB.*Mdr2*^{-/-} and BALB/c.*Mdr2*^{-/-} mice (right). One tick mark in time scale represents five seconds. **B:** BALB/c.*Mdr2*^{-/-} (right), but not FVB.*Mdr2*^{-/-} (left), mice demonstrate pronounced sinusoidal fibrosis (arrows). Data are means ± SEM (**A**). *n* = 3 to 9 per group (**A**). ****P* < 0.001 compared to FVB.*Mdr2*^{-/-} mice, two-way analysis of variance, Bonferroni post-test. LVP, left ventricular pressure.

Figure 6 Aged *Mdr2(Abc4)*^{-/-} mutant mice on BALB/cAnNCrl background demonstrate progressive loss of liver function and cirrhosis. **A:** Connective tissue staining (Sirius Red) of 7-month-old *Mdr2*^{-/-} mice. Note massive periportal scarring and severe sinusoidal fibrosis within adjacent parenchyma in BALB/c.*Mdr2*^{-/-} mice. Images on the **right** are enlarged from the **boxed areas** in the images on the **left**. **B:** Collagen deposition in livers of aged FVB and BALBc mice with homozygous disruption of *Mdr2* at 7 and 12 months of age. **C:** Serum alkaline phosphatase (ALP) and total bilirubin are elevated in 7 and 12 months in BALB/c.*Mdr2*^{-/-} mice. Data are expressed as means $\pm$ SEM. $n = 4$ to 8 per group. ** $P < 0.01$, *** $P < 0.001$ compared to FVB.*Mdr2*^{-/-} mice, two-way analysis of variance, followed by Bonferroni post-test. Original magnifications: $\times 50$ (**A**, **left panels**); $\times 200$ (**A**, **right panels**).

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**).

BALB/c.*Mdr2*^{-/-} Mice Demonstrate an Early Onset of Severe Portal Hypertension

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,

Figure 7 Development of primary liver cancer is accelerated in aged BALB/c.*Mdr2*^{-/-} mice. **A:** Representative macroscopic findings in 12-month-old FVB.*Mdr2*^{-/-} and BALB/c.*Mdr2*^{-/-} mice. **Arrows** indicate visible tumors. **B:** Incidence (**left panel**) and multiplicity (**right panel**) of liver tumors in both strains. **C:** Histological assessment of liver tumors confirms hepatocellular carcinoma. Representative serial sections of the tumor-fibrotic liver interface [hematoxylin and eosin stain (**top panels**) and reticulin stain (**bottom panels**)]. Data are expressed as means $\pm$ SEM. ** $P < 0.01$ compared to FVB.*Mdr2*^{-/-} mice (two-way analysis of variance, followed by Bonferroni post test). Original magnification, $\times 200$ (**C**). ND, no data.

4.9 ± 0.2 mmHg); however, significant differences between *Mdr2*^{-/-} strains were found at every time point starting from 4 weeks of age (Figure 5A). Portal pressure in BALB/c.*Mdr2*^{-/-} mice increased at a significantly higher pace compared to parental FVB.*Mdr2*^{-/-} mice ($P < 0.001$) (Figure 5A), peaking at 12 mmHg at 12 weeks (compared to 8.2 mmHg in FVB.*Mdr2*^{-/-} 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.*Mdr2*^{-/-} mice starting at 8 weeks, but not in age-matched FVB.*Mdr2*^{-/-} mice (Figure 5B), which can possibly be responsible for early-onset, severe portal hypertension in BALB/c.*Mdr2*^{-/-} mice.

Development of Cirrhosis and Primary Liver Cancer Is Accelerated in Aged BALB/c.*Mdr2*^{-/-} Mice

Finally, we evaluated the long-term liver disease outcomes in BALB/c.*Mdr2*^{-/-} and FVB.*Mdr2*^{-/-} mice at 7 and 12 months of age. Compared to the parental strain, BALB/c.*Mdr2*^{-/-} 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.*Mdr2*^{-/-} mice was severalfold higher at 7 months compared to FVB.*Mdr2*^{-/-} 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.*Mdr2*^{-/-} mice compared to FVB.*Mdr2*^{-/-} mice at 7 and 12 months of age (Figure 6C). Macroscopic examination of livers revealed that BALB/c.*Mdr2*^{-/-} mice developed visible liver tumors (>1 mm) starting at 7 months of age (Figure 7A and Supplemental Table S4), whereas tumors in FVB.*Mdr2*^{-/-} mice were only detected at 12 months. The overall tumor burden was also significantly higher in BALB/c.*Mdr2*^{-/-} 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).²⁷

Discussion

Herein, we generated and characterized a novel congenic *Mdr2*^{-/-} mouse on a fibrosis-susceptible background. Direct comparison of BALB/c.*Mdr2*^{-/-} mice with parental strain (fibrosis-resistant FVB/NJ) demonstrates that genetic background potentially influences the resultant phenotype, with dramatic acceleration of liver fibrosis,

early-onset portal hypertension, and primary liver cancer in BALB/c.*Mdr2*^{-/-} 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 CCl₄-induced models to achieve robust progressive fibrosis in resistant C57Bl/6 strain, through more frequent administration of escalating doses of hepatotoxins.²⁸ Hillebrandt et al⁸ reported remarkable differences in seven inbred mouse strains in CCl₄-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 *Mdr2*^{-/-} mice backcrossed onto BALB/cAnNCrl background showed an approximately threefold increase in resultant liver fibrosis compared to parental FVB.*Mdr2*^{-/-} (Figure 2C). Quantitatively, extensive fibrosis in BALB/c.*Mdr2*^{-/-} 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 µg per liver per week of hydroxyproline deposited in male BALB/c.*Mdr2*^{-/-} mice compared to 67 µg per liver per week in age- and sex-matched FVB.*Mdr2*^{-/-} 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 CCl₄ in C57Bl/6 mice, demonstrates a progression rate of 77 µg 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.*Mdr2*^{-/-} mice revealed that >40% of collagen is highly cross-linked (Figure 2D), compared to 20% in parental FVB.*Mdr2*^{-/-} and 27% in the CCl₄-induced model.²¹ Future studies should determine to what extent increased collagen cross-linking contributes to the susceptibility to fibrosis of BALB/c.*Mdr2*^{-/-} mice.

Previously, the Lammert laboratory reported generation of *Mdr2*^{-/-} 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.*Mdr2*^{-/-} strain was not performed, objective fibrosis parameters indicate that BALB/cJ.*Mdr2*^{-/-} strain, in terms of fibrotic response, is an improvement over fibrosis-resistant FVB, but develops less fibrosis than our new BALB/c.*Mdr2*^{-/-} mouse. The reported gold standard quantitative assessment of fibrosis

via hepatic hydroxyproline content is $48.0 \pm 7.6 \mu\text{g}/100 \text{ mg}$ in BALB/c.J.*Mdr2*^{-/-} mice at 8 weeks of age,³¹ compared to $70.3 \pm 3.8 \mu\text{g}/100 \text{ mg}$ 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/cJ and BALB/cAnNCrI 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/cAnNCrI 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*^{-/-} mouse, all tumors in both strains were classified as hepatocellular carcinoma by an experienced pathologist (I.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/cAnNCrI 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 profibrotic 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 (Col1 mRNA), on one hand, and down-regulation of profibrotic collagenase (Mmp-13) and concomitant overexpression of its inhibitor, tissue inhibitor of metalloproteinase 1, on the other hand (Figure 3).

Portal hypertension is one of the most important pathophysiological consequences of liver fibrosis. A hepatic venous pressure gradient >10 mmHg predicts cirrhosis decompensation^{35,36} 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/cAnNCrI 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.⁴¹ 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

1. Bataller R, Brenner DA: Liver fibrosis. *J Clin Invest* 2005, 115: 209–218
2. Friedman SL: Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008, 134:1655–1669
3. Schuppan D, Afdhal NH: Liver cirrhosis. *Lancet* 2008, 371:838–851
4. Talwalkar JA, Lindor KD: Primary sclerosing cholangitis. *Inflamm Bowel Dis* 2005, 11:62–72
5. Friedman SL, Sheppard D, Duffield JS, Violette S: Therapy for fibrotic diseases: nearing the starting line. *Sci Transl Med* 2013, 5: 167sr161
6. Patsenker E, Popov Y, Stickel F, Schneider V, Ledermann M, Sagesser H, Niedobitek G, Goodman SL, Schuppan D: Pharmacological inhibition of integrin α v β 3 aggravates experimental liver fibrosis and suppresses hepatic angiogenesis. *Hepatology* 2009, 50:1501–1511
7. Bissell DM: Therapy for hepatic fibrosis: revisiting the preclinical models. *Clin Res Hepatol Gastroenterol* 2011, 35:521–525

8. Hillebrandt S, Goos C, Matern S, Lammert F: Genome-wide analysis of hepatic fibrosis in inbred mice identifies the susceptibility locus *Hfib1* on chromosome 15. *Gastroenterology* 2002, 123:2041–2051
9. Hillebrandt S, Wasmuth HE, Weiskirchen R, Hellerbrand C, Keppeler H, Werth A, Schirin-Sokhan R, Wilkens G, Geier A, Lorenzen J, Kohl J, Gressner AM, Matern S, Lammert F: Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. *Nat Genet* 2005, 37:835–843
10. Shi Z, Wakil AE, Rockey DC: Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses. *Proc Natl Acad Sci U S A* 1997, 94:10663–10668
11. Trauner M, Fickert P, Wagner M: MDR3 (ABCB4) defects: a paradigm for the genetics of adult cholestatic syndromes. *Semin Liver Dis* 2007, 27:77–98
12. Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM, Borst P: Homozygous disruption of the murine MDR2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* 1993, 75:451–462
13. Fickert P, Fuchsbichler A, Wagner M, Zollner G, Kaser A, Tilg H, Krause R, Lammert F, Langner C, Zatloukal K, Marschall HU, Denk H, Trauner M: Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in *Mdr2* (*Abcb4*) knockout mice. *Gastroenterology* 2004, 127:261–274
14. Lammert F, Wang DQ, Hillebrandt S, Geier A, Fickert P, Trauner M, Matern S, Paigen B, Carey MC: Spontaneous cholecysto- and hepatolithiasis in *Mdr2*^{-/-} mice: a model for low phospholipid-associated cholelithiasis. *Hepatology* 2004, 39:117–128
15. Mauad TH, van Nieuwkerk CMJ, Dingemans KP, Smit JJM, Schinkel AH, Notenboom RGE, van den Bergh Weerman MA, Verkuisen RP, Groen AK, Oude Elferink RPJ, van der Valk MA, Borst P, Offerhaus GJA: Mice with homozygous disruption of the *mdr2* P-glycoprotein gene: a novel animal model for studies of non-suppurative inflammatory cholangitis and hepatocarcinogenesis. *Am J Pathol* 1994, 145:1237–1245
16. Fickert P, Zollner G, Fuchsbichler A, Stumptner C, Weiglein AH, Lammert F, Marschall HU, Tsybrovskyy O, Zatloukal K, Denk H, Trauner M: Ursodeoxycholic acid aggravates bile infarcts in bile duct-ligated and *Mdr2* knockout mice via disruption of cholangioles. *Gastroenterology* 2002, 123:1238–1251
17. Popov Y, Patsenker E, Fickert P, Trauner M, Schuppan D: *Mdr2* (*Abcb4*)^{-/-} mice spontaneously develop severe biliary fibrosis via massive dysregulation of pro- and antifibrogenic genes. *J Hepatol* 2005, 43:1045–1054
18. Gonzales E, Davit-Spraul A, Baussan C, Buffet C, Maurice M, Jacquemin E: Liver diseases related to MDR3 (ABCB4) gene deficiency. *Front Biosci (Landmark Ed)* 2009, 14:4242–4256
19. Ziolk M, Barbu V, Rosmorduc O, Frassati-Biaggi A, Barget N, Hermelin B, Scheffer GL, Bennouna S, Trinchet JC, Beaugrand M, Ganne-Carrie N: ABCB4 heterozygous gene mutations associated with fibrosing cholestatic liver disease in adults. *Gastroenterology* 2008, 135:131–141
20. Van Nieuwkerk CM, Elferink RP, Groen AK, Ottenhoff R, Tytgat GN, Dingemans KP, Van Den Bergh Weerman MA, Offerhaus GJ: Effects of Ursodeoxycholate and cholate feeding on liver disease in FVB mice with a disrupted *mdr2* P-glycoprotein gene. *Gastroenterology* 1996, 111:165–171
21. Popov Y, Sverdlov DY, Sharma AK, Bhaskar KR, Li S, Freitag TL, Lee J, Dieterich W, Melino G, Schuppan D: Tissue transglutaminase does not affect fibrotic matrix stability or regression of liver fibrosis in mice. *Gastroenterology* 2011, 140:1642–1652
22. Popov Y, Sverdlov DY, Bhaskar KR, Sharma AK, Millonig G, Patsenker E, Krahenbuhl S, Krahenbuhl L, Schuppan D: Macrophage-mediated phagocytosis of apoptotic cholangiocytes contributes to reversal of experimental biliary fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2010, 298:G323–G334
23. Popov Y, Patsenker E, Stickel F, Zaks J, Bhaskar KR, Niedobitek G, Kolb A, Friess H, Schuppan D: Integrin α v β 6 is a marker of the progression of biliary and portal liver fibrosis and a novel target for antifibrotic therapies. *J Hepatol* 2008, 48:453–464
24. Ferrell LD, Crawford JM, Dhillon AP, Scheuer PJ, Nakanuma Y: Proposal for standardized criteria for the diagnosis of benign, borderline, and malignant hepatocellular lesions arising in chronic advanced liver disease. *Am J Surg Pathol* 1993, 17:1113–1123
25. van Nieuwkerk CM, Groen AK, Ottenhoff R, van Wijland M, van den Bergh Weerman MA, Tytgat GN, Offerhaus JJ, Oude Elferink RP: The role of bile salt composition in liver pathology of *mdr2* (^{-/-}) mice: differences between males and females. *J Hepatol* 1997, 26:138–145
26. Milani S, Herbst H, Schuppan D, Stein H, Surrenti C: Transforming growth factors β 1 and β 2 are differentially expressed in fibrotic liver disease. *Am J Pathol* 1991, 139:1221–1229
27. Bergman S, Graeme-Cook F, Pitman MB: The usefulness of the reticulin stain in the differential diagnosis of liver nodules on fine-needle aspiration biopsy cell block preparations. *Mod Pathol* 1997, 10:1258–1264
28. Popov Y: Mouse model of primary biliary cirrhosis with progressive fibrosis: are we there yet? *Hepatology* 2013, 57:429–431
29. Issa R, Zhou X, Constandinou CM, Fallowfield J, Millward-Sadler H, Gaca MD, Sands E, Suliman I, Trim N, Knorr A, Arthur MJ, Benyon RC, Iredale JP: Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology* 2004, 126:1795–1808
30. Hillebrandt S, Keppeler H, Roderfeld M, Roeb E, Sauerbruch T, Lammert F: BALB-*Abcb4* knockout mice as new model for spontaneous liver fibrogenesis: genetic strain differences determine severity of fibrosis. *Hepatology* 2005, 42:599A
31. Henkel C, Roderfeld M, Weiskirchen R, Berres ML, Hillebrandt S, Lammert F, Meyer HE, Stuhler K, Graf J, Roeb E: Changes of the hepatic proteome in murine models for toxically induced fibrogenesis and sclerosing cholangitis. *Proteomics* 2006, 6:6538–6548
32. Roderfeld M, Rath T, Voswinckel R, Dierkes C, Dietrich H, Zahner D, Graf J, Roeb E: Bone marrow transplantation demonstrates medullar origin of CD34⁺ fibrocytes and ameliorates hepatic fibrosis in *Abcb4*^{-/-} mice. *Hepatology* 2010, 51:267–276
33. Blizard DA, Vandenbergh DJ, Jefferson AL, Chatlos CD, Vogler GP, McClearn GE: Effects of periadolescent ethanol exposure on alcohol preference in two BALB substrains. *Alcohol* 2004, 34:177–185
34. Seetharamaiah GS, Land KJ: Differential susceptibility of BALB/c and BALB/cBy mice to Graves' hyperthyroidism. *Thyroid* 2006, 16: 651–658
35. Berzigotti A, Seijo S, Reverter E, Bosch J: Assessing portal hypertension in liver diseases. *Expert Rev Gastroenterol Hepatol* 2013, 7:141–155
36. Ripoll C, Groszmann RJ, Garcia-Tsao G, Bosch J, Grace N, Burroughs A, Planas R, Escorsell A, Garcia-Pagan JC, Makuch R, Patch D, Matloff DS: Hepatic venous pressure gradient predicts development of hepatocellular carcinoma independently of severity of cirrhosis. *J Hepatol* 2009, 50:923–928
37. Abiralde JG, Tarantino I, Turnes J, Garcia-Pagan JC, Rodes J, Bosch J: Hemodynamic response to pharmacological treatment of portal hypertension and long-term prognosis of cirrhosis. *Hepatology* 2003, 37:902–908
38. Villanueva C, Lopez-Balaguer JM, Aracil C, Kolle L, Gonzalez B, Minana J, Soriano G, Guarner C, Balanzo J: Maintenance of hemodynamic response to treatment for portal hypertension and influence on complications of cirrhosis. *J Hepatol* 2004, 40:757–765
39. Turnes J, Garcia-Pagan JC, Abiralde JG, Hernandez-Guerra M, Dell'Era A, Bosch J: Pharmacological reduction of portal pressure and long-term risk of first variceal bleeding in patients with cirrhosis. *Am J Gastroenterol* 2006, 101:506–512
40. Patin E, Kutalik Z, Guernon J, Bibert S, Nalpas B, Jouanguy E, et al; International Hepatitis C Genetics Consortium; French ANRS HC EP 26 Genoscan Study Group: Genome-wide association study identifies variants associated with progression of liver fibrosis from HCV infection. *Gastroenterology* 2012, 143:1244–1252.e1–12
41. Friedman SL: Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol* 2010, 7:425–436