Fibromodulin, an Oxidative Stress-Sensitive Proteoglycan, Regulates the Fibrogenic Response to Liver Injury in Mice

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BACKGROUND & AIMS: Collagen I deposition contributes to liver fibrosis, yet little is known about other factors that mediate this process. Fibromodulin is a liver proteoglycan that regulates extracellular matrix organization and is induced by fibrogenic stimuli. We propose that fibromodulin contributes to the pathogenesis of fibrosis by regulating the fibrogenic phenotype of hepatic stellate cells (HSCs). METHODS: We analyzed liver samples from patients with hepatitis C–associated cirrhosis and healthy individuals (controls). We used a coculture model to study interactions among rat HSCs, hepatocytes, and sinusoidal endothelial cells. We induced fibrosis in livers of wild-type and Fmod−/− mice by bile duct ligation, injection of CCl4, or administration of thioacetamide. RESULTS: Liver samples from patients with cirrhosis had higher levels of fibromodulin messenger RNA and protein than controls. Bile duct ligation, CCl4, and thioacetamide each increased levels of fibromodulin protein in wild-type mice. HSCs, hepatocytes, and sinusoidal endothelial cells produced and secreted fibromodulin. Infection of HSCs with an adenovirus that expressed fibromodulin increased expression of collagen I and α-smooth muscle actin, indicating increased activation of HSCs and fibrogenic potential. Recombinant fibromodulin promoted proliferation, migration, and invasion of HSCs, contributing to their fibrogenic activity. Fibromodulin was sensitive to reactive oxygen species. HepG2 cells that express cytochrome P450 2E1 produced fibromodulin, and HSCs increased fibromodulin production in response to pro-oxidants. In mice, administration of an antioxidant prevented the increase in fibromodulin in response to CCl4. Coculture of hepatocytes or sinusoidal endothelial cells with HSCs increased the levels of reactive oxygen species in the culture medium, along with collagen I and fibromodulin proteins; this increase was prevented by catalase. Fibromodulin bound to collagen I, but the binding did not prevent collagen I degradation by matrix metalloproteinase 13. Bile duct ligation caused liver fibrosis in wild-type but not Fmod−/− mice. CONCLUSIONS: Fibromodulin levels are increased in livers of patients with cirrhosis. Hepatic fibromodulin activates HSCs and promotes collagen I deposition, which leads to liver fibrosis in mice.

Keywords: Mouse Model; Liver Disease; Intercellular Communication; Fibers.

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; BDL, bile duct ligation; ECM, extracellular matrix; FMOD, fibromodulin; HSC, hepatic stellate cell; IHC, immunohistochemistry; rFMOD, recombinant fibromodulin; ROS, reactive oxygen species; SEC, sinusoidal endothelial cell; TAA, thioacetamide; WT, wild-type.

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in defining tissue integrity. Moreover, in vivo both collagen and FMOD are likely to have an important functional role in tissues where they are coexpressed due to potential physical interaction between both proteins.

Thus far, there is no information on whether FMOD is present in the liver, which cells express it, and where specifically it is induced upon the establishment of hepatic injury. In addition, little is known about its potential role in the development of liver fibrosis, whether induction of FMOD in liver cells could contribute to the profibrogenic potential of HSCs, and the molecular mechanism involved in these events.

Identifying if FMOD stimulates the profibrogenic response to hepatic damage could be central for understanding the pathogenesis of liver fibrosis. Thus, the aim of this study was to explore how FMOD regulates the HSC profibrogenic phenotype, a key event in liver fibrosis, of this study was to explore how FMOD regulates the standing of the fibrogenic response to hepatic damage could be central for understanding the pathogenesis of liver fibrosis. Thus, the aim of this study was to explore how FMOD regulates the HSC profibrogenic phenotype, a key event in liver fibrosis, of this study was to explore how FMOD regulates the standing of the fibrogenic response to hepatic damage could be central for understanding the pathogenesis of liver fibrosis.

Materials and Methods

Cell Treatments

Rat HSCs (250,000 cells/well) were seeded on 6-well plates in Dulbecco’s modified Eagle medium/F12 with 10% fetal bovine serum. Primary cells were cultured using Dulbecco’s modified Eagle medium/F12 for 4 to 7 days, which was replaced by serum-deprived Dulbecco’s modified Eagle medium/F12 before treatment with 50 nmol/L endotoxin-free human recombinant FMOD (rFMOD) for 24 hours (donated by Dr Ake Oldberg, Lund University, Lund, Sweden). Cells were infected with Ad-LUC or Ad-FMOD at a multiplicity of infection of 50 for 48 hours. The adenoviruses were a gift from Dr David T. Curiel (Washington University, St Louis, MO). H2O2 (25 μmol/L) and catalase (200 U/mL) were added to the cells for 24 hours (both from Sigma, St Louis, MO).

Mice

FMOD−/− mice and their WT littermates (C57BL/6J) were obtained from Dr Marian Young (National Institutes of Health, Bethesda, MD). These mice were backcrossed for at least 10 generations. Colonies were established by intercrossing FMOD−/− mice, and littermates were used in all experiments. FMOD−/− mice have normal heart, liver, lung, skin, and cartilage; however, they show abnormal tissue organization, collagen fiber bundles, and fiber architecture.

Induction of Liver Injury

Ten-week-old male WT mice and their FMOD−/− littermates were used in all experiments. To induce liver injury, 3 in vivo models were used. In the first model, cholestasis was induced by placing a ligature around the common bile duct while controls were sham operated. All mice were killed 3 weeks later.

In the second model, mice were intraperitoneally injected twice a week with 0.5 mL/kg body wt CCl4 (Sigma) or an equal volume of mineral oil for 1 month and killed 48 hours after the last injection of CCl4. In the third model, mice were treated with thioacetamide (TAA) (300 mg/L; Sigma) in the drinking water or received an equal volume of water for 4 months. Mice were killed 48 hours after withdrawal of TAA. Blood was collected by orbital venous plexus bleeding. Each liver was excised into fragments by using the same liver lobe for biochemical assays and paraffin embedding for staining. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

Human Samples

Dr Andrea D. Branch (Mount Sinai School of Medicine, New York, NY) provided the human liver protein lysates and RNA from resections from de-identified controls and subjects with biopsy-proven stage 3 hepatitis C virus (HCV) cirrhosis. Samples were scored according to the Scheuer/Ludwig Batts classification. These samples were exempt from institutional review board approval because no patient information was disclosed.

Pathology

In all experiments, the left liver lobe was excised and fixed in 10% neutral-buffered formalin and processed into paraffin sections for H&E or immunohistochemistry (IHC) and scoring by the Brunt classification. Portal and lobular inflammation were noted to be lymphocytes present in the lobules or portal areas and were scored as follows: 1 = rare foci; 2 = up to 5 foci; 3 = >5 foci. Centrilobular necrosis and parenchymal necrosis were each separately scored. The scores for centrilobular necrosis were as follows: 1 = hepatocyte necrosis affecting only zone 3; 2 = in addition to zone 3 necrosis, occasional bridging necrosis was seen; 3 = pronounced bridging and confluent necrosis. Parenchymal necrosis was noted to be spotty necrosis or apoptosis in zones 2 and 1. The scores for parenchymal necrosis were as follows: 1 = ≤1 focus; 2 = 5–10 foci; 3 = ≥10 foci at 100x. Ductular reaction was noted to be proliferation of bile ductules at the margins of the portal tracts, and the score was as follows: 1 = rare bile ductules present; 2 = irregular buds of bile ductules affecting some portal tracts; 3 = when bile ductules are more prominent and affect the majority of portal areas and/or strings of

Figure 1. FMOD expression increases in patients with HCV-induced cirrhosis. (A) Patients with biopsy-proven stage 3 HCV-induced cirrhosis showed an increase in FMOD and collagen I protein levels compared with healthy individuals. Likewise, there was a significant elevation in FMOD mRNA levels in patients with stage 3 HCV-induced cirrhosis compared with healthy liver explants. (B) The bar graph represents the fold change in FMOD mRNA normalized by that of GAPDH. n = 5; **P < .01 and ***P < .001 for HCV-induced cirrhosis vs healthy explants.
bile ductular epithelial cells were seen intermingled with hepatocytes. The degree of fibrosis ranged from 0 to 4 and was patterned after the Brunt system. Briefly, this was as follows: 1 = perisinusoidal/perivenular fibrosis alone; 2 = 1 plus portal fibrosis; 3 = bridging fibrosis; 4 = cirrhosis. The assessment of the preceding scores was uniformly performed under 100× magnification in 10 fields per sample and twice.

**Immunohistochemistry**

The collagen I antibody used on IHC was from Millipore (Billerica, MA), the FMOD and cytokeratin-19 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), the α-smooth muscle actin (α-SMA) antibody was from Sigma, and the desmin antibody was from Dako (Carpinteria, CA). The FMOD antibody was tested in livers from Fmod−/− mice and competed with rFMOD (2.5 μg rFMOD and 2 μg FMOD antibody in 1 mL were incubated for 30 min at room temperature) to ensure specificity (Supplementary Figure 1). Reactions were developed using the Histostain Plus Detection System (Invitrogen, Carlsbad, CA). For the Sirius red computer-assisted morphometry assessment, the integrated optical density was calculated from 10 random fields per sample containing similar-size portal tract or hepatic vein at 100× and using Image-Pro 7.0 Software (Media Cybernetics, Bethesda, MD). The results were averaged and expressed as fold change over controls.

**Statistical Analysis**

Data were analyzed by a 2-factor analysis of variance, and results are expressed as mean ± SEM. All in vitro experiments were performed in triplicate at least 4 times. A representa-
Results

**FMOD Increases in Patients With Stage 3 HCV-Induced Cirrhosis**

To determine whether FMOD is expressed in human livers, resections from healthy patients and patients with biopsy-proven stage 3 HCV-induced cirrhosis were studied for FMOD protein and messenger RNA (mRNA) expression. Western blot analysis showed an increase in both FMOD and collagen I protein levels in patients with HCV-induced cirrhosis compared with healthy explants (Figure 1A). Likewise, there was an 8-fold increase in FMOD mRNA levels in patients with HCV-induced cirrhosis compared with healthy explants (Figure 1B). Hence, these results confirm that FMOD is expressed in human liver and that it is up-regulated in stage 3 HCV-induced cirrhosis.

**Expression of FMOD Is Up-regulated in Cholestasis and in Drug-Induced Liver Injury in Mice**

To dissect if FMOD also increased during liver injury in mice, we used well-established in vivo models of liver fibrosis such as common bile duct ligation (BDL), which causes cholestatic liver damage, or long-term CCl4 injection and TAA treatment to provoke drug-induced liver injury. These 2 drugs undergo cytochrome P450 metabolism, leading to significant oxidant stress, inflammation, and pericentral necrosis.

FMOD protein was induced under chronic common BDL (Figure 2A), CCl4 injection (Figure 2B), and TAA treatment (Figure 2C). In all 3 models, FMOD expression was detected mostly in the sinusoids and in hepatocytes. FMOD protein expression in the BDL, CCl4, and TAA models was also validated by Western blot analysis (Figure 2D). Thus, there was an association between FMOD protein up-regulation and the extent of liver injury in mice.
To identify the specific cell type that expressed FMOD, chronic CCl₄-injured WT mouse livers were perfused and primary cells were isolated. Western blot analysis revealed that HSCs, hepatocytes, and SECs expressed and secreted FMOD protein (Figure 2E and F); however, Kupffer cells did not express FMOD (not shown).

**FMOD Activates and Induces Profibrogenic Effects in HSCs**

Because FMOD protein was found induced in HSCs during liver injury, we hypothesized that endogenous FMOD could enhance activation of HSCs and their profibrogenic potential. To show this, rat HSCs were infected with Ad-LUC or Ad-FMOD. Adenoviral infection did not alter HSC viability or phenotype (Figure 3A, left). Along with enhanced intracellular FMOD expression, intracellular collagen I and to some extent α-SMA, a marker of HSC activation, were increased by Ad-FMOD compared with Ad-LUC infection (Figure 3A, right). Hence, an autocrine role for intracellular FMOD in modulating collagen I deposition and HSC activation could be established.

Because the IHC analysis also identified hepatocytes and SECs as sources of FMOD, and FMOD was secreted by all 3 cell types, next we questioned whether extracellular FMOD could play a role in regulating the profibrogenic phenotype of HSCs, which entails their proliferative, migratory, and invasive potential. To evaluate this, rat HSCs were challenged with rFMOD and cell proliferation, migration, and invasion were evaluated over time. rFMOD induced HSC proliferation by 35%, as shown by the rate of methyl[³H]-thymidine incorporation into the DNA of HSCs (Figure 3B). Migration, measured by wound closure, was also enhanced by rFMOD treatment (Figure 3C, arrows), and it was not blocked by preincubation with mitomycin to inhibit mitosis (not shown). Lastly, HSC invasion or chemotaxis was increased by 6-fold in the presence of rFMOD when compared with nontreated cells (Figure 3D and E). Overall, these findings show that FMOD also exerts a paracrine role in regulating the profibrogenic behavior and phenotype of HSCs.

**ROS Increase FMOD Protein Expression**

Because cholestasis and drug-induced liver injury generate a significant amount of ROS due to glutathione depletion and increased ROS generation, mostly via cytochrome P450 2E1 activation, next we evaluated whether FMOD was ROS sensitive. First, HepG2 cells overexpressing cytochrome P450 2E1, as a source of ROS, were analyzed for FMOD expression. Western blot analysis showed significant up-regulation of both cytochrome P450 2E1 and intracellular and extracellular FMOD proteins (Figure 4A). In addition, primary rat HSCs incubated with H₂O₂ displayed greater intracellular and extracellular FMOD protein expression than nontreated HSCs (Figure 4B).

To validate the induction of FMOD by oxidative stress in vivo, WT mice were injected with CCl₄ for 1 month in the presence or absence of S-adenosylmethionine, an antioxidant known to restore glutathione levels, or mineral oil. Coinjection with S-adenosylmethionine lowered by 50% FMOD protein levels (Supplementary Figure 2A) and the extent of liver fibrosis when compared with mice injected with CCl₄ alone. Overall, these data indicate that FMOD is an ROS-sensitive proteoglycan. Because collagen I is highly inducible by oxidant stress, FMOD could be a potential mediator regulating collagen I deposition.

**Hepatocytes and SECs Increase FMOD and Collagen I Levels in HSCs in Coculture**

Because in vivo models of cholestasis or drug-induced liver injury identified hepatocytes and SECs as sources of FMOD in addition to HSCs, which were responsive to rFMOD, to analyze whether these cells also contributed to the fibrogenic response in a paracrine fashion, cocultures with HSCs were established. Primary rat HSCs were placed in coculture with either primary SECs or hepatocytes. Coculture with either cell type led to an increase in ROS levels (mostly hydroperoxides) in the cell culture medium compared with HSCs cultured alone. As anticipated, the increase in ROS was rather significant...
in the hepatocyte coculture compared with the SEC coculture (Figure 5A); hence, based on our previous studies showing the effect of hepatocyte-derived ROS generation on the HSC profibrogenic behavior,9,29–32 we prioritized this coculture model.

Coculture of HSCs with SECs increased both intracellular FMOD and collagen I expression compared with the HSC monoculture (Figure 5B). In contrast, coculture of HSCs with hepatocytes notably elevated both intracellular and extracellular collagen I and FMOD expression in the HSC and hepatocyte coculture and HSC monoculture (Figure 5C). Thus, these results show that FMOD from neighboring cells could also signal to enhance the HSC profibrogenic response.

Lastly, because the coculture of HSCs with hepatocytes showed the highest increase in ROS levels, based on previous work,9,29–32 to determine whether ROS could be upstream of FMOD, the hepatocyte and HSC coculture was incubated in the presence of an antioxidant. Catalase blocked the effect of the cocultures on FMOD, collagen I, and α-SMA. Results were expressed as average values. Experiments were performed in triplicate 4 times. *P < .05, **P < .01, and ***P < .001 for coculture vs monoculture; **P < .001 for catalase vs control.

**BDL Induces More Liver Injury and Fibrosis in WT Than in Fmod−/− Mice**

Because the interaction between collagen I and FMOD could increase the stability of collagen I, hence favoring scarring, samples from cocultures of HSCs with hepatocytes were immunoprecipitated with anti-FMOD antibody and immunoblotted with anti-collagen I antibody. There was binding of FMOD and collagen I proteins (Supplementary Figure 3A). Infection of HSCs with Ad-FMOD to overexpress FMOD and treatment with active matrix metalloproteinase 13, a key HSC protease known to degrade collagen I within the triple-helical structure,33 did not alter collagen I degradation compared with HSCs infected with Ad-LUC and treated with active matrix metalloproteinase 13 (Supplementary Figure 3B). Thus, the FMOD/collagen I binding in HSCs did not increase collagen I stability against matrix metalloprotease 13 proteolytic degradation.

**FMOD Binds Collagen I but Does Not Prevent Collagen I Degradation by Matrix Metalloproteinase 13**

Because the interaction between collagen I and FMOD could increase the stability of collagen I, hence favoring scarring, samples from cocultures of HSCs with hepatocytes were immunoprecipitated with anti-FMOD antibody and immunoblotted with anti-collagen I antibody. There was binding of FMOD and collagen I proteins (Supplementary Figure 3A). Infection of HSCs with Ad-FMOD to overexpress FMOD and treatment with active matrix metalloproteinase 13, a key HSC protease known to degrade collagen I within the triple-helical structure,33 did not alter collagen I degradation compared with HSCs infected with Ad-LUC and treated with active matrix metalloproteinase 13 (Supplementary Figure 3B). Thus, the FMOD/collagen I binding in HSCs did not increase collagen I stability against matrix metalloprotease 13 proteolytic degradation.
or sham operated and killed 3 weeks later. Serum γ-glutamyltransferase activity increased in BDL compared with sham-operated WT mice, whereas lower activity was observed in Fmod−/− mice (Figure 6A). H&E staining showed that coagulative necrosis, inflammation, and ductular reaction were present in BDL WT mice but were less apparent in Fmod−/− mice (Figure 6B). The scores for necrosis, portal inflammation, lobular inflammation, and ductular reaction validated these findings (Figure 6C–F). Lastly, IHC analysis depicted more desmin, collagen I deposition, Sirius red staining and morphometry, cytokeratin-19 (a marker for ductular reaction), and neutrophil staining in BDL WT than in Fmod−/− mice. The Brunt pathology scores for necrosis, portal inflammation, lobular inflammation, and ductular reaction were shown in panels C to F. CV, central vein; PV, portal vein. n = 6/group; *P < .05, **P < .01, and ***P < .001 for BDL vs sham; ●P < .05 and ●●P < .01 for Fmod−/− BDL vs BDL WT.

WT Mice Show More CCl4-Induced Chronic Liver Injury and Fibrosis Than Fmod−/− Mice

To dissect whether Fmod ablation could be protective in a model of chronic drug-induced liver injury affecting mostly the central zone, 10-week-old male WT and Fmod−/− mice were subjected to common BDL or sham operation and were killed 3 weeks later. The activity of γ-glutamyltransferase is shown in panel A. (B) H&E staining demonstrating greater necrosis (blue arrows), inflammation (yellow arrow), and ductular reaction (green arrows) in BDL WT than in Fmod−/− mice. The Brunt pathology scores for necrosis, portal inflammation, lobular inflammation, and ductular reaction are shown in panels C to F. CV, central vein; PV, portal vein. n = 6/group; *P < .05, **P < .01, and ***P < .001 for BDL vs sham; ●P < .05 and ●●P < .01 for Fmod−/− BDL vs BDL WT.

Discussion

The overall goal of this study was to determine the potential role of FMOD on the profibrogenic phenotype of HSCs and its contribution to the fibrogenic response to liver injury. Although previous work by Krull et al.19 did not identify Fmod mRNA in normal rat liver, our studies show that FMOD protein is expressed in human liver and is significantly elevated in patients with stage 3 HCV-induced cirrhosis compared with healthy individuals. Induction of FMOD has been previously described in bleomycin-induced pulmonary fibrosis in rats,34 and FMOD gene transcription is induced by UV irradiation.35 In addition, we show that FMOD expression is up-regulated in cholestasis in the BDL model and in drug-induced liver injury in the CCl4 and TAA models in WT mice.

FMOD-positive staining was identified mostly in parenchymal and in sinusoidal areas. Specificity of the FMOD staining on IHC was further confirmed by using Fmod−/− mice by competing with rFMOD and by isolating primary cells from CCl4-treated WT mice. Hepatocytes, SECs, and HSCs, but not Kupffer cells, expressed FMOD protein.
Figure 7. WT mice show more BDL-induced fibrosis, ductular reaction, and neutrophil infiltration than Fmod<sup>-/-</sup> mice. (A) IHC depicted more desmin and collagen I protein in BDL WT mice than in Fmod<sup>-/-</sup> mice (pink arrows). (B and C) Sirius red/fast green staining and morphometry (pink arrows). (D) IHC for cytokeratin-19 (green arrows). (E) Naphthol AS-D Chloroacetate Esterase (NADSCA) staining to demonstrate the presence of neutrophils (pink arrows). CV, central vein; PV, portal vein. n = 6/group. Results are expressed as average values. **<i>P</i> < .01 and ***<i>P</i> < .001 for BDL vs sham and ●●●<i>P</i> < .001 for Fmod<sup>-/-</sup> vs WT.
Thus, we speculated that the effects of FMOD on HSCs would be both of autocrine and paracrine nature because HSCs were exposed to secreted FMOD produced by themselves and by neighboring liver cells.

To evaluate the autocrine effects of FMOD on the HSC profibrogenic phenotype, intracellular FMOD was induced by infecting HSCs with Ad-FMOD. This resulted in an increase in intracellular and extracellular collagen I along with α-SMA, thus validating an autocrine role for FMOD in driving HSC activation and in promoting their profibrogenic potential.

To dissect whether extracellular FMOD could also condition the HSC behavior, cells were incubated in the presence of rFMOD, which caused an increase in HSC proliferation, migration, and invasive potential. These are important functions gained by HSCs during their activation process and greatly contribute to their profibrogenic ability. Thus, the experimental data suggest that FMOD activates and induces a profibrogenic response in HSCs in both an autocrine and a paracrine manner.

Oxidative stress represents a common link among different modes of persistent liver injury. Because FMOD is expressed in hepatocytes, SECs, and HSCs and FMOD was found to be ROS sensitive in vitro and in vivo, also described by others in fibroblasts, we speculated that communication between these cell types could lead to an enhanced profibrogenic behavior in HSCs. Several studies, including our own, have evaluated the role of conditioned medium from hepatocytes and SECs in stimulating HSCs. These studies have identified ROS as key mediators for the profibrogenic actions on HSCs.

To date, there is limited information on the specific ROS-sensitive mediators secreted by hepatocytes, SECs, and HSCs, as well as the molecular mechanisms by which these molecules modulate the fibrogenic response in HSCs. Thus, a coculture model was developed to study the role of hepatocytes and SEC-secreted FMOD on HSC activation and collagen I production. These models resemble aspects of the cross talk of hepatocytes and SECs with HSCs in vivo and have been previously used to gain mechanistic insight on the communication between these cell types. A novel role of FMOD as an ROS downstream effector on collagen I up-regulation and HSC activation was identified, which was blocked by catalase, an antioxidant.

Thus, FMOD could also convey paracrine-mediated signaling to regulate the profibrogenic behavior of HSCs; however, whether these effects require receptor binding and further intracellular signaling in HSCs still remains to be determined because the FMOD receptors, if any, have not been identified so far. In addition, FMOD did not exert its profibrogenic effects by modulating transforming growth factor β, a well-known profibrogenic factor, because Western blot analysis showed similar transforming growth factor β expression in BDL Fmod−/− and in WT mice. Our studies also ruled out that the physical interaction between FMOD and collagen I could prevent the matrix metalloproteinase 13-mediated collagen I proteolysis in HSCs, thus contributing to ECM deposition.

All these results suggested that Fmod gene ablation could protect from the onset of liver fibrosis. The in vivo data showed that in the chronic BDL model, where significant periportal fibrosis occurs due to biliary hyperplasia, Fmod−/− mice developed less coagulative necrosis, inflammation, biliary epithelial cell proliferation, ductular reaction, and neutrophil infiltration than WT mice. Moreover, collagen I expression was lower in BDL Fmod−/− compared with WT mice, and comparable findings were obtained in mice treated with CCl4, which also depicted lower α-SMA induction.

In conclusion, our results identified FMOD as expressed in the liver and induced upon the onset of chronic liver injury. Furthermore, they suggest a role for the ROS-driven FMOD increase in regulating HSC activation and their profibrogenic potential both in an autocrine and a paracrine fashion; hence, FMOD could be considered as a new target to prevent the development and progression of liver fibrosis.

**Supplementary Material**

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.11.029.

**References**


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Acknowledgments
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Conflicts of interest
The authors disclose no conflicts.

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**Supplementary Materials and Methods**

**General Methodology**

HSC proliferation was calculated from the rate of methyl[^3H]-thymidine incorporation into the DNA of HSCs. Details on general methodology such as H&E staining, Sirius red/fast green staining, Naphthol AS-D Chloroacetate Esterase staining for neutrophils, adenoviral infection, extracellular ROS measurement, and immunoprecipitation/immunoblotting have been described in previous publications.\(^1\) Cytochrome P450–expressing cell line steadily generates ROS due to sustained cytochrome P450 E1 uncoupling.\(^7\)\(^8\)

**Primary Cell Isolation**

Primary HSCs were isolated from male Sprague-Dawley rats (500 ± 25 g) by a 2-step in situ liver perfusion with pronase and Liberase Blendzyme 3 (Roche, Indianapolis, IN). Hepatocytes were separated from non-parenchymal cells, centrifuging for 3 minutes at 50g. HSCs were collected by density gradient centrifugation in 11% over 17.5% Histodenz (Sigma). Cell purity (>95%) was assessed by cellular UV emission at 350 nm. SECs were separated from the nonparenchymal cell fraction by centrifugal elutriation at 18 mL/min as previously described.\(^3\) In some experiments, HSCs that had been passed less than 3 times were used. Mice were perfused by using a protocol similar to that for rats.

**Coculture Model**

Details on the coculture setting have been described in earlier publications.\(^3\)\(^5\)\(^6\) The only modification was the incorporation of isolated SECs to the transwells. Cell ratios were similar to those found in the liver. After 1 day of incubation, the medium was removed and the transwells containing the rat hepatocytes or SECs were transferred onto the rat HSC wells for 1 day. The control and cytochrome P450–expressing HepG2 cell lines were donated by Dr Arthur I. Cederbaum (Mount Sinai School of Medicine, New York, NY). The cytochrome P450–expressing cell line steadily generates ROS due to sustained cytochrome P450 E1 uncoupling.\(^7\)\(^8\)

**Wound Healing In Vitro Assay**

Rat HSCs were seeded and grown to confluence, after which a mechanical wound was made on the center of the culture with a sterile 200-μL pipette tip. Lifted cells were removed using serum-free DMEM/F12, and 0 to 50 nmol/L rFMOD was added in serum-free DMEM/F12 for 24 hours. Experiments were also performed in cells pretreated with 10 μmol/L mitomycin for 1 hour to inhibit cell proliferation. A series of multiple pictures of the wounds were captured at 200X by using an inverted microscope.

**HSC Invasion**

For the invasion or chemotaxis assay, we used a modified transwell cell culture chamber. The outer surface of an 8-μm transwell was coated with rat collagen I for 1 hour under sterile conditions. Rat HSCs (25,000 cells/well) were seeded in serum-free DMEM/F12 on the upper chamber, and the lower chamber was filled with serum-free DMEM/F12 plus 0 to 50 nmol/L rFMOD. After 24 hours, the nonmigrating HSCs on the upper surface of the filter were removed with a cotton swab and the cells invading the lower side of the filter in the transwells were fixed in ice-cold methanol and stained with H&E. The filters with fixed cells were detached from the transwells and mounted on glass slides. The number of HSCs present in 10 random fields at 100X was quantified as mean number of migrating cells.

**Quantitative Reverse-Transcription Polymerase Chain Reaction**

Liver RNA was extracted using the RNaseasy Mini Kit (Qiagen, Chatsworth, CA) and treated with deoxyribonuclease. RNA (1 μg) was reverse transcribed using first-strand complementary DNA synthesis with random primers (Promega, Madison, WI). Quantitative real-time polymerase chain reaction was performed in a Roche Light Cycler 480 with the following polymerase chain reaction primers: FMOD 5′-ACCACTTGATAGTGAGGAC-3′, FMOD 3′-GACCCGTGAGATCTGTGTG-5′, GAPDH 5′-CATGAGCCCTTTCACTAGAC-3′ and GAPDH 3′-GATCTCGCTCCTGGAAGATG-5′. The specific target expression was calculated by using the cycle threshold method. Each sample was amplified in triplicate, and each gene was analyzed on samples from at least 3 individual experiments. Values were normalized to GAPDH.

**Western Blot Analysis**

Details on the Western blot methodology can be found in previous work from our laboratory.\(^1\)\(^6\) The FMOD, actin, and calnexin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the α-SMA antibody was from Sigma, and the transforming growth factor β antibody was from R&D Systems (Minneapolis, MN). Anti-CYP2E1 antibody was a gift from Dr Jerome M. Lasker (Puracyp Inc, Carlsbad, CA). Intracellular FMOD is detected as 3 bands of ~59, ~42, and ~36 kDa, and extracellular FMOD appears as one single band of ~59 kDa due to glycosylation of the protein. Anti-rat collagen I was provided by Dr Detlef Schuppan (Harvard Medical School, Boston, MA). The anti-rat collagen I antibody detects pro-collagen α1(I) and α2(I) and N-terminally processed procollagen I, which run at ~165 to 200 kDa, as well as collagen I α1 and α2 chains, which run at ~135 kDa. Human collagen I was detected by using an antibody from Millipore (Billerica, MA) that mostly detects collagen I α1 and α2 chains, which run at ~135 kDa. Intracellular collagen I and FMOD refer to...
proteins detected in cells after removing the cell culture medium and washing with phosphate-buffered saline twice; thus, some protein bound to the cells may still remain. Extracellular collagen I and FMOD refer to secreted unbound protein, precipitated with acetone (1:9, vol/vol) overnight and collected after centrifugation at 11,000g for 30 minutes. The quantification under the blots refers to the sum of bands from all collagen I and FMOD isoforms.

The enhanced chemiluminescence reaction was developed by using the Las4000 scanner (Fujifilm, Stamford, CT). The intensity of the Western blot bands was quantified using the National Institutes of Health Image J software. All Western blots were performed in triplicate from at least 4 different experiments. All samples from each experiment were run on the same gel and transferred onto the same nitrocellulose membrane. Extracellular proteins analyzed by Western blot were corrected by total protein content, and protein loading was subsequently verified by Ponceau red staining on each nitrocellulose membrane. The loading controls for intracellular proteins were calnexin or actin.

In Vivo Cotreatment With an Antioxidant

To determine the protective role of antioxidant treatment in vivo and its effects on FMOD expression, WT mice were injected intraperitoneally with CCl4 or CCl4 plus S-adenosylmethionine (SAM) for 1 month. SAM was administered at a dose of 10 mg/kg body wt daily and was always given 2 hours before the CCl4 injection. Control groups received mineral oil or mineral oil plus SAM.

Collagen I Proteolysis

HSCs were infected with Ad-LUC or Ad-FMOD for 48 hours to allow FMOD overexpression as well as collagen I and FMOD in vitro binding. Samples of non-infected cells were used as controls for total collagen content. Matrix metalloproteinase 13 (MMP13) was pre-activated by treatment with 2.5 mmol/L p-aminophenyl-mercuric acetate for 30 minutes. Cell lysate and culture medium were then incubated with preactivated MMP13 for 1, 2, 6, and 24 hours to allow for collagen I degradation in the presence or absence of overexpressed FMOD. The reactions were stopped by addition of 15 μmol/L EDTA. Samples were neutralized, and a Western blot analysis for collagen I was performed. Collagen I proteolysis was detected by the disappearance of the high-molecular-weight bands corresponding to the α1 and α2 collagen I and the appearance of lower-molecular-weight bands in the blot.

Supplementary References

Supplementary Figure 1. Specificity of the FMOD antibody. To show that the FMOD antibody used on IHC and Western blot analysis was specific, livers from Fmod<sup>−/−</sup> mice injected with CCl<sub>4</sub> for 1 month were immunostained for FMOD. A representative sample is shown in panel A. Likewise, livers from WT mice injected with CCl<sub>4</sub> for 1 month were immunostained for FMOD in the presence or absence of rFMOD as a competitor for the binding reaction (B).
**Supplementary Figure 2.** (A) SAM protects WT mice from CCl₄-induced FMOD induction. WT mice were injected mineral oil, SAM, mineral oil plus CCl₄, or SAM plus CCl₄ for 1 month. Cotreated mice showed decreased FMOD expression, which was quantified by Western blot analysis. Results are expressed as mean values ± SEM. n = 6/group; **P < .01 and ***P < .001 for SAM plus CCl₄ vs mineral oil plus CCl₄.

(B) Transforming growth factor β expression is similar in BDL WT and Fmod⁻/⁻ mice. Ten-week-old male WT and Fmod⁻/⁻ mice were subjected to common BDL or sham operation and were killed 3 weeks later. Western blot analysis showing similar transforming growth factor β in fibrotic livers from WT and Fmod⁻/⁻ mice. n = 6/group; ***P < .001 for BDL vs sham.

**Supplementary Figure 3.** FMOD binds collagen I but does not prevent collagen I degradation by MMP13. (A) Samples from the cocultures of HSCs with hepatocytes were immunoprecipitated with anti-FMOD antibody and immunoblotted with anti–collagen I antibody. HSCs were infected with Ad-LUC or Ad-FMOD to allow FMOD overexpression along with collagen I and FMOD binding. Samples from noninfected cells were used as controls to determine the amount of collagen I present in the absence of FMOD. Cell lysate and culture medium were incubated with preactivated MMP13 in a time course experiment to allow for collagen I degradation in the presence or absence of overexpressed FMOD. Reactions were stopped by addition of 15 μmol/L ethylenediaminetetraacetic acid; after neutralization, a Western blot analysis for collagen I was performed. The 2-hour time point is shown in the blot. (B) Collagen I proteolysis was detected by the disappearance of high-molecular-weight collagen I bands (arrowheads) and the appearance of lower-molecular-weight bands in the blot (arrows and ellipses).
Supplementary Figure 4. WT show more CCl4-induced chronic liver injury and fibrosis than Fmod−/− mice. Ten-week-old male WT and Fmod−/− mice were injected intraperitoneally with CCl4 or mineral oil for 1 month and were killed 48 hours after the last injection. (A) H&E staining revealed more inflammation (yellow arrows) and necrosis (blue arrows) in CCl4-injected WT than in Fmod−/− mice. The inflammation and necrosis scores according to the Brunt classification are shown in panel B. (C) Likewise, collagen I IHC showed greater collagen I deposition in CCl4-injected WT than in Fmod−/− mice (pink arrows). (D and E) Sirius red/fast green staining and morphometry also showed greater collagen deposition in CCl4-injected WT than in Fmod−/− mice (pink arrows). (F) α-SMA IHC (pink arrows). Results are expressed as mean values ± SEM. n = 6/group; **p < .01 and ***p < .001 for Fmod−/− vs WT.