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Cholestatic liver fibrosis is caused by obstruction of the biliary tract and is associated with early activation of portal fibroblasts (PFs) that express Thy-1, fibulin 2, and the recently identified marker mesothelin (MSLN). Here, we have demonstrated that activated PFs (aPFs) and myofibroblasts play a critical role in the pathogenesis of liver fibrosis induced by bile duct ligation (BDL). Conditional ablation of MSLN+ aPFs in BDL-injured mice attenuated liver fibrosis by approximately 50%. Similar results were observed in MSLN-deficient mice (Msln–/– mice) or mice deficient in the MSLN ligand mucin 16 (Muc16–/– mice). In vitro analysis revealed that MSLN regulates TGF-β1–inducible activation of WT PFs by disrupting the formation of an inhibitory Thy-1–TGFβRI complex. MSLN also facilitated the FGF-mediated proliferation of WT aPFs. Therapeutic administration of anti-MSLN–blocking Abs attenuated BDL-induced fibrosis in WT mice. Liver specimens from patients with cholestatic liver fibrosis had increased numbers of MSLN+ aPFs/myofibroblasts, suggesting that MSLN may be a potential target for antifibrotic therapy.
Mesothelin/mucin 16 signaling in activated portal fibroblasts regulates cholestatic liver fibrosis

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Cholestatic liver fibrosis is caused by obstruction of the biliary tract and is associated with early activation of portal fibroblasts (PFs) that express Thy-1, fibulin 2, and the recently identified marker mesothelin (MSLN). Here, we have demonstrated that activated PFs (aPFs) and myofibroblasts play a critical role in the pathogenesis of liver fibrosis induced by bile duct ligation (BDL). Conditional ablation of MSLN+ aPFs in BDL-injured mice attenuated liver fibrosis by approximately 50%. Similar results were observed in MSLN-deficient mice (Msln−/− mice) or mice deficient in the MSLN ligand mucin 16 (Muc16−/− mice). In vitro analysis revealed that MSLN regulates TGF-β1-inducible activation of WT PFs by disrupting the formation of an inhibitory Thy-1-TGFβRI complex. MSLN also facilitated the FGF-mediated proliferation of WT aPFs. Therapeutic administration of anti-MSLN–blocking Abs attenuated BDL-induced fibrosis in WT mice. Liver specimens from patients with cholestatic liver fibrosis had increased numbers of MSLN+ aPFs/myofibroblasts, suggesting that MSLN may be a potential target for antifibrotic therapy.

Introduction

Fibrosis is an aberrant wound-healing process, in which chronic injury in any tissue activates myofibroblasts to produce a fibrous scar (1). Myofibroblasts are rare in normal tissues, and their origin depends on the tissue and type of injury. Many types of research suggest that liver-resident hepatic stellate cells (HSCs) and portal fibroblasts (PFs) are major sources of myofibroblasts in fibrotic liver (2). However, the composition of myofibroblasts varies significantly, depending on the etiology of hepatic fibrosis; hepatotoxic liver fibrosis (injury to hepatocytes, such as that caused by chronic viral hepatitis and nonalcoholic steatohepatitis) results from the activation of HSCs (3), while activated PFs (aPFs) are implicated in the pathogenesis of cholestatic fibrosis (obstruction to bile flow, such as that caused by primary and secondary biliary cirrhosis and primary sclerosing cholangitis) (4).

PFs are “periductular mesenchymal cells” that maintain the integrity of the portal tract (5, 6). In response to biliary obstruction, portal (myo)fibroblasts proliferate, upregulate expression of collagen type I, α-smooth muscle actin (α-SMA), TGF-β1/2, IL-6, and IL-13, and give rise to myofibroblasts (2, 7–14). Using a flow cytometry–based method of aPF purification (from bile duct ligation [BDL]–injured collagen-α1[I]–GFP reporter mice, which express GFP under the control of the Colla1 promoter) (4), we demonstrated that myofibroblasts derived from aPFs (referred to herein as aPFs/myofibroblasts) are a major source of collagen type I–producing cells in early cholestatic liver injury, contributing more than 70% of the myofibroblasts by day 5 after BDL. The relative contribution of aPFs/myofibroblasts decreases with progressive injury, as HSCs become activated and contribute to the myofibroblast population (17 and 20 days after BDL). aPFs/myofibroblasts express high levels of collagen type I, serve as a significant source of TGF-β2, secrete IL-13 and IL-6 (4, 6), and facilitate the ductular reaction (cholestasis-induced proliferation of bile ducts) (7). aPFs are activated by taurocholic acid and IL-25, but unlike activated HSCs (aHSCs), are unresponsive to PDGF (4). aPFs can be distinguished from aHSCs by expression of Thy-1, fibulin 2, elastin, and the ecto-ATPase nucleoside triphosphohydrolase 2 (NTPD2) and by their lack of aHSC markers (retinoid, desmin, glial fibrillary acidic protein [GFAP], p75NGFR) (15–18). Gene expression profiling of freshly isolated aPFs/myofibroblasts has identified several novel markers of aPFs, including mesothelin (MSLN) and mucin 16 (MUC16) (4).

MSLN (19), a membrane-anchored glycosylphosphatidylinositol–linked (GPI-linked) 71-kDa membrane protein (MSLN precursor), is a mesothelial cell marker that is proteolitically cleaved to generate mature MSLN (40 kDa) and megakaryocyte-potentiating factor (MPF) (30 kDa) (20). Both molecules appear to be biologically active, although their exact function remains unknown. MSLN is expressed in several human malignancies, including mesotheliomas and ovarian cancer. Studies of patients with ovarian cancer have identified the cancer antigen CA125 as an MSLN ligand (20–23), which is widely used as a diagnostic marker (with the exception of pregnancy and liver cirrhosis, which are considered false-positives) (24). Since high expression of MSLN is linked to increased tumor proliferation and invasion, MSLN is a novel
Cholestatic fibrosis in mice. Mechanistic studies identified what we believe to be a novel TGF-β1 pathway, in which interaction of MSLN with a MUC16–Thy-1–TGFβRI complex regulates TGF-β1-mediated activation of aPFs/myofibroblasts. Analysis of patients’ liver biopsies revealed that MSLN+Thy-1+ aPFs contribute to cholestatic fibrosis in patients with secondary biliary fibrosis and biliary atresia (BA), suggesting that MSLN may serve as an attractive target for antifibrotic therapy.

**Results**

Msln-KO mice are resistant to BDL-induced cholestatic, but not hepatotoxic, liver fibrosis. To test the role of MSLN in the activation of aPFs/myofibroblasts, WT and Msln<sup>−/−</sup> mice were subjected to BDL and CCl₄ injury (Figure 1A–C). We observed no difference in liver fibrosis in CCl₄-injured WT or Msln<sup>−/−</sup> mice (at 1.5 months, Figure 1B). In concordance with this finding, the aPF markers MSLN and fibulin 2 were not upregulated in the livers of CCl₄-injured mice.
control diet–fed WT mice) (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI88845DS1). We observed significant downregulation of Picrosirius red staining (↓1.7-fold decrease) and expression of the fibrogenic genes \(\text{Col1a1} \) (↓2-fold), \(\alpha\)-SMA (↓2-fold), and \(\text{Timp1} \) (↓1.7-fold) in the livers of \(\text{Msln}^{-/-}\) mice (Supplemental Figure 1A), which was associated with decreased expression of the aPF-specific gene fibulin 2. Our data indicate that MSLN critically contributes to the pathogenesis of cholestatic liver fibrosis of different etiologies.

The number of BDL-activated myofibroblasts was reduced in \(\text{Msln}^{-/-}\) mice. To further assess the role of MSLN in PF/myofibroblast activation, WT and \(\text{Msln}^{-/-}\) mice were crossed with reporter collagen \(\alpha\)-1(I)–GFP mice (in which all activated myofibroblasts upregulate collagen \(\alpha\)-1(I)–GFP expression) and subjected to sham or BDL treatment (5 and 17 days of BDL), Figure 2, A and B, and Supplemental Figure 1, B–D). Livers were costained with Thy-1 and desmin to visualize aPF- and aHSC-derived myofibroblasts, respectively (Figure 2A), and the area of Thy-1+GFP+ and Thy-1–GFP+ costaining was calculated (Figure 2B). Although expression of collagen \(\alpha\)-1(I)–GFP was increased in both WT and \(\text{Msln}^{-/-}\) mice following BDL (5 days of BDL vs. sham-operated mice), the number of activated GFP + myofibroblasts was reduced by nearly 3-fold in BDL-injured \(\text{Msln}^{-/-}\) mice (1.6% ± 0.2% vs. 4.8% ± 0.2% in BDL-treated WT mice). Meanwhile, the composition of GFP + myofibroblasts (considered 100%) was not significantly changed between WT and \(\text{Msln}^{-/-}\) mice after 5 days of BDL. Thus, WT GFP + myofibroblasts (Figure 1C), confirming that aPFs/myofibroblasts do not activate in response to hepatotoxic liver injury.

In contrast, the development of BDL-induced cholestatic liver fibrosis was strongly attenuated in \(\text{Msln}^{-/-}\) mice compared with that seen in WT mice (Figure 1, A–C). We observed significant downregulation of Picrosirius red staining (2-fold) and mRNA expression of the fibrogenic genes \(\text{Col1a1} \) (2-fold), \(\alpha\)-SMA (1.7-fold), and \(\text{Timp1} \) (1.7-fold) in the livers of \(\text{Msln}^{-/-}\) mice 5 days after BDL, which correlated with low levels of serum alanine transaminase (ALT) and aspartate transaminase (ALP) (vs. levels in WT mice). We also observed inhibition of liver fibrosis in \(\text{Msln}^{-/-}\) mice on day 17 after BDL, as shown by decreased Picrosirius red staining (↓1.8-fold) and downregulation of \(\text{Col1a1} \) (↓1.8-fold), \(\alpha\)-SMA (↓1.4-fold), and \(\text{Timp1} \) (↓1.7-fold) mRNA expression, as well as reduced levels of serum ALP (↓2-fold). Our data indicate that MSLN critically contributes to the pathogenesis of cholestatic liver fibrosis of different etiologies.

Msln-KO mice are resistant to diethoxycarbonyl dihydrocollidine–induced cholestatic fibrosis. Similar results were obtained when WT and \(\text{Msln}^{-/-}\) mice were subjected to another model of cholestatic liver injury induced by 3 weeks of diethoxycarbonyl dihydrocollidine (DDC) feeding. As expected, hepatic expression of \(\text{Msln} \) mRNA was strongly increased in DDC-fed WT mice (7-fold increase vs. control diet–fed WT mice) (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI88845DS1). We observed significant downregulation of Picrosirius red staining (↓1.7-fold decrease) and expression of the fibrogenic genes \(\text{Col1a1} \) (↓2-fold), \(\alpha\)-SMA (↓2-fold), and \(\text{Timp1} \) (↓1.7-fold) in the livers of \(\text{Msln}^{-/-}\) mice (Supplemental Figure 1A), which was associated with decreased expression of the aPF-specific gene fibulin 2. Our data indicate that MSLN critically contributes to the pathogenesis of cholestatic liver fibrosis of different etiologies.

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Figure 3. Ablation of Msln attenuates development of cholestatic liver fibrosis in mice. (A) Tamoxifen administration (12 × 5 mg/mouse by oral gavage) irreversibly labeled all MSLN+ aPFs by nuclear LacZ (nLacZ) expression in WT MslnnLacZ mice (n = 14) and caused ablation of aPFs in MslnDTA littermates (C57BL/6, 8-week-old male mice, n = 17 from 3 independent experiments). Livers were stained for LacZ (micrographs show gross liver tissue) and (B) analyzed for LacZ expression (original magnification, ×40). (C) Liver sections were immunostained with DAPI for Thy-1 and α-SMA expression. The number of aPFs in sham-operated MslnnLacZ and MslnDTA mice and the efficiency of aPF/myofibroblast ablation in BDL-operated MslnDTA mice were calculated in comparison with the number of Thy-1+α-SMA+DAPI+ cells in livers of BDL-MslnnLacZ mice (considered as 100%). Representative micrographs are shown (original magnification, ×20). (D) Livers were stained with Picrosirius red (original magnification, ×4). (E) Quantification of Picrosirius red+ and Thy-1+α-SMA+ areas is shown as a percentage. Expression of fibrogenic, aPF-specific, and inflammatory gene mRNA was analyzed by qPCR and is shown as a fold induction. *P < 0.05 and **P < 0.01, by 2-tailed Student’s t test. (See also Supplemental Figure 3.)

were composed of 71% ± 3% aPFs and 28% ± 2% aHSCs, while MSLN-deficient GFP+ myofibroblasts were composed of 61% ± 2% aPFs and 38% ± 4% aHSCs.

Expression of MSLN has been reported in both aPFs/myofibroblasts and hepatic mesothelial cells, identified by expression of the GPM6A marker (34). Costaining with anti–glycoprotein M6A (anti-GPM6A) Ab revealed that mesothelial cells contribute to a small number of collagen α1(I)–GFP-positive (Col+) myofibroblasts residing in the liver capsule of the WT mice subjected to 5 days of BDL injury (Supplemental Figure 2A), while the majority of Thy-1+Col+ myofibroblasts were located in the portal areas and lacked GPM6A expression (Supplemental Figure 2B). Since no other cell types in the liver (including HSCs, cholangiocytes, endothelial cells, hepatocytes, and Kupffer cells) (Supplemental Figure 2, C and D) were reported to coexpress collagen α1(I) and MSLN (and Thy-1), the loss of activated myofibroblasts in BDL-injured Msln−/− mice was attributed to the MSLN deficiency in aPFs/myofibroblasts.
Ablation of MSLN+ aPFs attenuates BDL-induced liver fibrosis. MSLN was minimally expressed in adult mice but was strongly upregulated in response to BDL (Figure 1C). To gain insight into the role of MSLN+ aPFs/myofibroblasts in the pathogenesis of cholestatic liver fibrosis, we generated mice devoid of MSLN+ cells. We assumed that, if MSLN+ aPFs significantly contribute to cholestatic liver fibrosis, then ablation of MSLN+ aPFs would attenuate the development of cholestatic liver fibrosis. To examine this, MslnDTA mice (MslnnLacZ mice, in which the cassette harboring the reporter nLacZ and tamoxifen-inducible ER-Cre was knocked into the Msln gene) were crossed with Rosa26 flox-Stop-flox-DTA mice (Figure 3A). The resulting MSLN-ablated Msln DTA mice and WT MslnnLacZ littermates were treated with tamoxifen and then subjected to 5 days of BDL injury. Upon administration of tamoxifen to MslnnLacZ mice, MSLN+ aPFs/myofibroblasts had increased nLacZ+ expression and were scattered throughout the liver parenchyma of MslnnLacZ mice (Figure 3B and Supplemental Figure 3A). In turn, the disappearance of hepatic nLacZ+ expression in Msln DTA mice was indicative of successful ablation of MSLN+ aPFs/myofibroblasts and correlated with marked downregulation of the aPF-specific markers MSLN and fibulin 2 in the livers of Msln DTA mice (Figure 3E). DAPI costaining for overlapping expression of α-SMA and Thy-1 confirmed that deletion of more than 80% of the Thy-1+α-SMA+ myofibroblasts was achieved in BDL-MslnDTA mice (vs. BDL-treated MslnnLacZ mice) (Figure 3C and Supplemental Figure 3B).

We compared the development of cholestatic fibrosis in MslnDTA and WT MslnnLacZ mice 5 days after BDL surgery and found that ablation of MSLN+ aPFs/myofibroblasts attenuated liver fibrosis by approximately 50%, as demonstrated by the reduced area of Picrosirius red staining and reduced expression of Col1a1, α-SMA, and Timp1 mRNA (Figure 3, D and E). We concluded that MSLN-expressing aPFs/myofibroblasts contribute to cholestatic liver fibrosis and that therapeutic targeting of MSLN+ aPFs/myofibroblasts might attenuate BDL-induced fibrosis in mice.

Delayed administration of anti-MSLN Abs inhibits cholestatic fibrosis in mice. To test whether anti-MSLN-blocking Abs can avert the development of cholestatic fibrosis, BDL-operated collagen α1(I)-GFP mice were treated with 2 consecutive doses of anti-MSLN Abs or isotype IgG (24 and 48 hours after BDL) (Figure 4A) and sacrificed 5 days after BDL surgery. Delayed administration of Ab no. 1 (Ab1) (200 ng/g or 400 ng/g BW; Abbiotec; 250S19) to BDL-treated collagen α1(I)-GFP mice inhibited fibrosis and activation of GFP+ myofibroblasts in a dose-dependent manner. Similar results were achieved using delayed administration of Ab no.
Expression of human MSLN is upregulated in patients with cholestatic liver fibrosis. To gain insight into the role of αPFs in human liver diseases, the myofibroblast composition was assessed in resected livers from patients with cholestatic fibrosis (BA, \( n = 6 \); secondary biliary cirrhosis, \( n = 12 \); and hepatotoxic HCV, \( n = 5 \)) using quantitative PCR (qPCR) using immunohistochemical analyses (Figure 4B and Supplemental Figure 4). Expression of MSLN mRNA was upregulated by nearly 3-fold in patients with cholestatic fibrosis (but not in those with nonfibrotic or HCV-fibrotic livers). Immunostaining for MSLN and Thy-1\(^+\) expression was detected only in fibrotic lesions of patients with cholestatic fibrosis and overlapped with \( \alpha\)-SMA\(^+\) (but not desmin\(^+\)) staining, suggesting that MSLN can serve as a marker of human αPFs (Supplemental Figure 4) and mouse αPFs (4).

Primary Msln\(^{-/-}\) αPFs/myofibroblasts have a defect in activation and proliferation. To investigate the role of MSLN in αPF/myofibroblast activation, primary αPFs were sort purified from BDL-injured (for 5 days) livers of collagen \( \alpha\)-I\(^1\)–GFP WT and collagen \( \alpha\)-I\(^1\)–GFP Msln\(^{-/-}\) mice and phenotyped (Supplemental Figure 5). In comparison with WT αPFs, we found that expression of fibrogenic genes was...
downregulated in Msln⁻/⁻ aPFs (fold decrease of 2.5 for Colla1, 2.5 for Colla2, 2.5 for α-SMA, and 1.5 for Timp1) (Figure 5A). Expression of cyclin D was also decreased (2.5-fold), suggesting that Msln⁻/⁻ aPFs might have a proliferative defect. Furthermore, we performed a scratch assay to examine the proliferation and migration of WT and Msln⁻/⁻ aPFs (Figure 5B) and found that, unlike WT aPFs, Msln⁻/⁻ aPFs failed to populate the scratch area within 12 hours and showed a defect in expression of the proliferation marker Ki67 (Figure 5C). Our data suggest that Msln-deficient aPFs/myofibroblasts exhibit defective activation and proliferation.

**Imortalized Msln⁻/⁻ aPFs exhibit defective TGF-β1 signaling.** Next, we investigated the role of Msln in the activation of aPFs/myofibroblasts using immortalized WT and Msln⁻/⁻ aPFs (generated by introducing SV40 large T antigen). Like primary Msln⁻/⁻ aPFs, immortalized Msln⁻/⁻ aPFs had defective TGF-β1 signaling. Induction of the TGF-β1 target genes Colla1, α-SMA, PAI-1, and activin (Figure 6A) was reduced in TGF-β1-stimulated Msln⁻/⁻ aPFs and was associated with impaired phosphorylation of the SMAD2/3 complex (Figure 6B) as compared with WT aPFs. In addition, Msln⁻/⁻ aPFs had upregulated expression of Thy-1 (150-fold), Mac6 (2.5-fold), and Smad7 (2-fold) mRNA (Figure 6A). Although previous studies have implicated Thy-1 (36) and Smad7 (37) in the blockade of TGF-β1 responses in tissue fibroblasts (38–42), the association of Msln and MUC16 with Thy-1 and Smad7 and their role in fibrogenesis has not been reported. Msln is a part of a TGFβRII signaling complex. To explore the relationship between Msln (31), Thy-1 (43), Smad7 (44), and MUC16 (33), we performed a series of IPs using WT and Msln⁻/⁻ aPFs, with or without TGF-β1 (Figure 6, C-E). IP with anti-TGFβRII Ab identified Thy-1, MUC16, and TGFβRII proteins as binding partners of TGFβRII in both WT and Msln⁻/⁻ aPFs (Figure 6C). Interaction between TGFβRII, Thy-1, and MUC16 was reduced in TGFβ1-stimulated WT and Msln⁻/⁻ aPFs, while binding of TGFβRII to TGFβRII was increased. In comparison with the WT aPFs, we observed that formation of a Thy-1–SMAD7–TGFβRII complex was increased in unstimulated Msln⁻/⁻ aPFs, while TGFβRII–MUC16 binding was reduced and normalized to the amount of precipitated TGFβRII (Figure 6E and Supplemental Figure 6A).
with reduced degradation of FGF receptor 1 (FGFR1) following FGF signaling (Figure 9C). Next, we performed IPs with anti-FGFR1 Ab and anti-MSLN Ab to determine the relationship between MSLN and FGFR1, but did not identify MSLN-MUC16 as binding partners of the FGFR1 complex (Figure 9D), suggesting that MSLN directly regulates AKT rather than the FGF signaling pathway.

Activation of JAK2-STAT3 and ERK1/2 regulates the proliferation of aPFs.

We further investigated the molecular mechanism of FGF-mediated aPF proliferation (Figure 10 and Supplemental Figure 8). As expected, we found that pretreatment with an inhibitor of FGFR1 completely blocked (100%, vs. DMSO) cyclin D mRNA expression in FGF-induced WT aPFs (Supplemental Figure 8). Cyclin D expression (Supplemental Figure 8) and STAT3 phosphorylation (Figure 10A) were also suppressed by inhibitors of AKT and JAK2, but most effectively (>80%) by AZD1480, an inhibitor of JAK2 and ERK (Figure 10B). In agreement with these results, the proliferation and migration of WT and Msln–/– aPFs to the scratch area were completely blocked by AZD1480 (Figure 10C) and associated with complete inhibition of ERK1/2 and STAT3 phosphorylation (Figure 10, B and C). Our proposed model of MSLN-MUC16-dependent regulation of FGF signaling in aPFs is depicted in Figure 10D.

Reconstitution of MSLN rescues the phenotype in Msln–/– cells.

To confirm these findings, we transfected Msln–/– aPFs with a pCMV-MSLN construct (Figure 11A and Supplemental Figure 9A). We observed that reexpression of MSLN restored basal and FGF-dependent proliferation in MslnKO+Msln aPFs, as shown by the

of TGF-β1 signaling in aPFs and that the MSLN-MUC16 complex might further facilitate cholestatic fibrosis (by an additional 25%, approximately) through the mediation of other important signaling pathways in aPFs. In the next set of experiments, we investigated the role of the MSLN/MUC16 signaling pathway in aPF proliferation.

Msln–/– aPFs exhibit defective FGF-induced proliferation. FGF was identified as an important factor that drives the proliferation of aPFs. Primary cultured WT aPFs had a growth advantage over Msln–/– aPFs (Figure 5, B and C). In an attempt to further characterize MSLN-MUC16 functions, we examined the proliferation and responses to FGF using immortalized WT and Msln–/– aPFs and found that cyclin D mRNA expression was suppressed by 4.5-fold in FGF-stimulated Msln–/– aPFs and correlated with the downregulation of Mmp9 mRNA (Figure 9A). When we examined the components of the FGF signaling pathway, we found that phosphorylation of AKT was strongly reduced (by 5-fold) in FGF-stimulated Msln–/– aPFs (Figure 9C). A novel mechanism for terminating RTK signaling and reducing receptor abundance has been described and showed that, in AKT-impaired cells, RTK expression and trafficking are defective, resulting in prolonged signaling and increased activation of ERK1/2 (45). In accordance with previously published reports, we detected an 11-fold increase in ERK1/2 phosphorylation in FGF-stimulated Msln–/– aPFs (Figure 9B) that was associated with reduced degradation of FGF receptor 1 (FGFRI) following FGF signaling (Figure 9C). Next, we performed IPs with anti-FGFR1 Ab and anti-MSLN Ab to determine the relationship between MSLN and FGFR1, but did not identify MSLN-MUC16 as binding partners of the FGFR1 complex (Figure 9D), suggesting that MSLN directly regulates AKT rather than the FGF signaling pathway.

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upregulation of cyclin D mRNA (Figure 11B) and the increased numbers of cells populating the scratch area (Figure 11C and Supplemental Figure 9B), both of which could be also blocked by AZD1480 (Supplemental Figure 9C). We found that reexpression of MSLN also restored Colla1 and Muc16 in MslnKO-Msk aPFs, but had no effect on the expression levels of Thy-1, which remained elevated, as observed in Msln+/− aPFs (Figure 11A).

Inhibition of Thy-1 restores TGF-β1–induced responses in Msln+/− cells. To test whether suppression of Thy-1 can restore the responsiveness of Msln+/− aPFs to TGF-β1, we transfected Msln+/− aPFs with Thy-1 siRNA or control siRNA (Figure 12A). Inhibition of Thy-1 restored phosphorylation of SMAD2 (Figure 9D) and the expression of Colla1 in TGF-β1–stimulated MslnKO-Thy-1 aPFs, but had no effect on cyclin D (Figure 12, A and B) or ERK1/2 (Supplemental Figure 10), suggesting that the regulatory function of Thy-1 is restricted to TGF-β signaling.

Overexpression of AKT restores Msln+/− cell proliferation. Furthermore, to test whether restoration of AKT phosphorylation can reinitiate the proliferation of Msln+/− aPFs, we infected Msln+/− aPFs with constitutively activated (CA) AKT (AAV-CA-AKT) or with a control adenoviral construct expressing GFP (AAV-GFP). We found that reexpression of MSLN also restored Col1a1 and Muc16 in MslnKO+Msln aPFs, but had no effect on the expression levels of Thy-1, which remained elevated, as observed in Msln+/− aPFs (Figure 11A).
lioma (26), and both MSLN and CA125 became targets for anti-
cancer therapy. Here, we demonstrate that expression of MSLN
is not restricted to tumorigenesis, but is strongly induced in aPFs
in response to cholestatic liver injury (Figure 1 and Supplemental
Figure 1A). In support of this, Msln
KO mice developed a defect in
the activation of cancer-associated myofibroblasts that resulted in
reduced tumorigenesis (25–29).

Despite extensive studies of human cancer cells, the func-
tional properties of the MSLN/MUC16 signaling pathway
remain poorly understood. MUC16 was identified as an MSLN
ligand, and MSLN/MUC16 signaling was linked to AKT and
ERK1/2 activation and was implicated in the metastatic growth
and dissemination of cancer cells (46–49). Like other GPI-an-
chored proteins, MSLN requires MUC16 and/or utilizes other
receptors for intracellular signaling. Our studies of immortal-
ized aPFs show that Msln–/– aPFs exhibit impaired proliferation
and responses to FGF and that these defects are associated with
defective AKT phosphorylation (Figure 9C). However, co-IP
experiments did not detect MSLN-MUC16 as a part of the FGF /
FGFR1 signaling complex. A novel mechanism of AKT activa-
tion has been identified that implicates AKT in the regulation
of RTK trafficking and degradation (45). Accordingly, cells
deficient in AKT activation fail to properly express EGFR and
PDGFR, but as a compensatory mechanism, strongly upregulate
ERK1/2 phosphorylation. We observed a similar phenotype in
Msln–/– aPFs (Figure 9B). The loss of AKT activation was asso-

Discussion
We demonstrate here that aPFs mediate hepatic fibrosis at the
onset of cholestatic injury and that ablation of MSLN+ aPFs in mice
decreases BDL-induced (5 days) liver fibrosis by approximately
50%. This study links MSLN/MUC16 signaling to the pathogen-
esis of cholestatic fibrosis and shows that deletion of MSLN and/
or MUC16 in mice reduces cholestatic liver fibrosis by nearly 50%.
We determined that MSLN facilitates both TGF-β1–induced activa-
tion of aPFs and FGF-induced proliferation of aPFs. We detect-
ed a similar upregulation of MSLN+ aPFs in patients with biliary
fibrosis of different etiologies. Our data demonstrate that therapeu-
tic administration of MSLN-blocking Abs attenuates BDL-
induced liver fibrosis in mice, suggesting that MSLN could serve as
a target for antifibrotic therapy.

MSLN (19) is highly expressed during embryonic develop-
ment (17, 32) but is downregulated in adulthood (20). However,
Msln–/– and Muc16–/– mice do not have developmental abnormali-
ties (31, 33). In turn, human MSLN and CA125 (the human coun-
terpart of MUC16) is highly upregulated in several malignancies
such as ovarian cancer, hepatocellular carcinoma, and mesothe-
associated with an inability of Msln−/− aPFs to mount FGF-mediated responses. Because of reduced phosphorylation of JAK2 (despite upregulation of JAK2 expression), we observed that FGF-dependent activation of STAT3 was also impaired in Msln−/− aPFs. In turn, we observed that basal and FGF-induced ERK1/2 activation was highly increased in Msln−/− aPFs and was driving the residual proliferative capability of Msln−/− aPFs (via phosphorylation of STAT3). Although the exact mechanism by which MSLN-MUC16 activates AKT remains unknown, our data indicate that the MSLN/MUC16/AKT pathway is a key regulator of cholestasis-induced proliferation of aPFs/myofibroblasts, but does not affect the fibrogenic properties of aPFs.
Msln−/− aPFs exhibit defective TGF-β1 signaling manifested by reduced expression of profibrogenic genes and TGF-β1 target genes (Figure 6A). Here, we demonstrate the involvement of MSLN in the TGF-β1 signaling pathway and propose a mechanism in which the MSLN-MUC16 complex interacts with the TGFβRI receptor and facilitates TGF-β1 signaling in WT aPFs via disruption of TGFβRII binding to its inhibitory receptor Thy-1 (Figure 6, C and D). This enables TGFβRI-TGFβRII interaction, phosphorylation and nuclear translocation of SMAD2/3/4, and transcription of target genes. In turn, depletion of Msln in Msln−/− aPFs resulted in overexpression of Thy-1 (>150-fold vs. WT levels), which binds to TGFβRI and prevents TGF-β1 signaling. Consistently, inhibition of Thy-1 partially restored TGF-β1 signaling in Msln−/− aPFs, but had no effect on ERK1/2 activation.

Like MSLN, Thy-1 is a GPI-anchored protein (50) that is expressed in fibroblasts, T cells, and neurons and is considered a specific marker for these cell types. Thy-1 was implicated in the inhibition of TGF-β1 responses in tissue fibroblasts via interaction with αvβ5 integrins (38–42). While Thy-1 functions are not well characterized, deletion of Thy-1 in mice was shown to exacerbate bleomycin-induced lung fibrosis (36). Our data suggest that Thy-1 blocks TGFβRI and that formation of the MSLN-Thy-1 complex enables TGF-β1 signaling. In accordance with this, downregulation of Thy-1 expression restored TGF-β1 signaling in Msln−/− mice, and deletion of Thy-1 in mice aggravated the development of cholestatic liver fibrosis. We identified TGFβRI as a binding partner of Thy-1 and MUC16 as a binding partner of MSLN. Since knocking out MSLN and MUC16 receptors together or separately yielded similar phenotypes in BDL-injured mice (Figure 7), we propose that the MSLN-MUC16 complex regulates the same signaling pathway in tissue fibroblasts and, therefore, can serve as a unique target for antifibrotic therapy in patients with cholestatic fibrosis.

Overall, our proposed model (summarized in Figure 6F and Figure 10D) suggests that the MSLN/MUC16 signaling pathway facilitates cholestatic fibrosis in mice via regulation of TGF-β1-mediated activation and FGF-induced proliferation of aPFs. In response to TGF-β1 signaling, MSLN disrupts the Thy-1-TGFβRI inhibitory complex and enables binding of TGF-β1 to its cognate receptor, phosphorylation and nuclear translocation of SMAD2/3, and transcription of the TGF-β1 target genes PAI1 and activin. MSLN and Thy-1 differentially regulate TGF-β1 signaling in aPFs, and MSLN serves as a functional repressor of Thy-1. This represents, to our knowledge, a novel level of regulation of TGF-β1 signaling that may have broad implications for tissue homeostasis, remodeling, and fibrosis in multiple organs. Furthermore, our data suggest that the MSLN-MUC16 complex regulates FGF-dependent proliferation of aPFs, although indirectly, via activation of AKT, which regulates the trafficking and degradation of FGFR1 (45).
In support of these findings, a role for MSLN and CA125 (human analog of murine MUC16) in the regulation of AKT phosphorylation has been suggested in human cancer cells and was associated with increased proliferation and metastatic dissemination of cancer cells (49). CA125 release has been documented in patients with various malignancies and now serves as a standard marker in the diagnosis of ovarian cancer in patients (51). The role of MSLN and MUC16 in patients with liver fibrosis has not been evaluated. However, elevated serum levels of MSLN and CA125 were observed clinically in patients with liver fibrosis, and these levels were considered nonsignificant (52). Here we provide, for the first time to our knowledge, evidence that expression of MSLN is upregulated in the livers of patients with cholestatic fibrosis (Figure 4B) and that MSLN might play a role in the pathogenesis of BA and primary sclerosing cholangitis and possibly serve as a target for antifibrotic therapy. Further investigations are required to evaluate the role of MSLN and MUC16 in patients with cholestatic fibrosis.

Although the origin of aPFs during development has been identified in embryonic mesenchyme (32), which is also rich in MSLN expression (31, 53), the source of proliferating aPFs in the adult injured murine liver remains unclear. In response to cholestatic liver injury, adult aPFs may result from endogenous mesenchymal progenitors (53), which were recently identified and implicated in smooth muscle cell and fibroblast replenishment. The presence of mesenchymal progenitors was also reported in other parenchymal organs (56–58). Indepen-
dent studies suggested that cardiac, hepatic, and lung fibroblasts arise from mesenchymal progenitors expressing Thy-1 (59), gremlin 1 (60), MSLN (53), CD9 (61), and CD105 (59) (and/or others), which are newly recognized progenitor mark-
ers associated with the self-renewal capability of adult mesenchymal cells. Remarkably, expression of the same markers was detected in aPFs (2, 4, 6). Given the high proliferative capacity of aPFs emerging in fibrogenic liver within days after BDL, we can speculate that aPFs might derive from hepatic mesothelial progenitors. Heterogeneity of the aPF/myofibroblast population cannot be excluded, and upregulation of appropriate markers in aPFs may vary, depending on the duration of BDL, the stage of activation/differentiation, and the localization within the hepatic lobule. In support of this, expression of MSLN, Thy-1, and fibulin 2 is induced in aPFs throughout BDL injury (Figure 1). And in agreement with previous findings (34), we observed that mesothelial cells, identified by expression of the GPM6A marker, also contributed to a small popula-
tion of Col1 myofibroblasts residing in the liver capsule of WT mice that had undergone 5 days of BDL injury (Supple-
mental Figure 2), while the majority of myofibroblasts were composed of Thy-1-Col1 aPFs/myofibroblasts, located in the portal areas, and desmin-Col1 aHSCs/myofibroblasts, located in the acinus. Concordant with our previous findings, aPFs/myofibroblasts contributed to 60% to 70% of hepatic myofi-
broblasts after BDL-induced (5 days) liver fibrosis (4).

**Methods**

**Patient specimens.** Archived liver specimens were obtained from 16 patients with liver cirrhosis (HCV, n = 5 [METAVIR score of F2]; obstructive jaundice, n = 5 [F2]; BA, n = 6) and from 3 patients who were undergoing liver resection for reasons unrelated to liver fibrosis (normal liver) at the Kyoto University Hospital (2010 to 2013).

**Mice and liver injury.** Eight-week-old collagen α1(I)-GFP mice (62), Msln−/− mice (31), Msln−/−/Muc16−/−/Col1a1−/− mice (53), Muc16−/− mice (33), Thy-1−/− mice (50), Rosa26CreER mTmG pups (The Jackson Laboratory; 009669), Smadfl/fl mice (The Jackson Laboratory; 017462), and WT littermates on a C57BL/6 background were maintained under specific pathogen–free conditions at UCSD and had ad libitum access to normal chow and water. Liver injury was induced in mice by ligation of the common bile duct (for 5 days and 17 days), or by oral gavage with CCl4 carbon tetrachloride CCl4 (1:4 dilution in corn oil, 60 μl × 14 injections) (4). An additional model of cholestatic liver fibrosis was induced in WT and Msln−/− mice fed a diethyoxycarbonyl dihydrocollidine (DDO) diet (AD5001; Custom Animal Diets) or a control diet (AD3012; Custom Animal Diets) for 3 weeks.

**Serum ALT and ALP measurements.** Serum ALT and ALP measurements were analyzed using Infinity ALT (Thermo Fisher Scientific), a QuantiChrom ALP Assay Kit (BioAssay Systems), and a VALIDATE calibration verification kit (Maine Standards Company LLC).

**Histology.** Formalin-fixed, paraffin-embedded human or mouse livers were stained with Picrosirius red (analyzed by a pathologist)
and LMP cell line (murine pancreatic MSLN + and MUC16 + cancer, blasts, gift of James S. Hagood, (UCSD, La Jolla, California, USA) Msln–/– AKT was overexpressed in Msln–/– knockdown was achieved in Msln–/– mice. We are grateful to Koji Taniguchi (Keio University, Tokyo, Japan) for his technical support of the manuscript. TK provided support, designed the study, and wrote the manuscript.

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53. Kinkevich Y, Mori T, Sahoo D, Xu PX, Bermingham JR, Weissman IL. Identification and prospective isolation of a mesothelial


