EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

Semaphorin 7A Contributes to TGF- β —Mediated Liver Fibrogenesis

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Semaphorin7A (SEMA7A) is a membrane-anchored protein involved in immune and inflammatory responses, exerting an effect on pulmonary fibrosis. Thus, we aimed to investigate the role of SEMA7A in hepatic fibrosis. Liver injury was induced *in vivo* by carbon tetrachloride i.p. injection or bile duct ligation in wild-type and SEMA7A knockout (KO) mice. Human and mouse liver samples and primary mouse hepatic cell populations were used for Western blot analysis, quantitative real-time RT-PCR, and immunohistochemistry. SEMA7A is highly expressed in hepatic stellate cells (HSCs). The expression of SEMA7A and its receptor β 1-integrin subunit increase during liver injury and in activated HSCs. Transforming growth factor β —stimulated HSCs showed increased expression of SEMA7A in a SMAD2/3-independent manner, leading to increased expression of fibrogenic and inflammation markers. This pattern was significantly blunted in SEMA7A KO HSCs. Overexpression of SEMA7A in HSCs showed increased fibrogenic and inflammation markers expression. *In vivo*, SEMA7A KO mice treated with carbon tetrachloride and bile duct ligation developed reduced fibrosis versus wild-type mice. Moreover, SEMA7A expression increased in liver samples of patients with fibrosis versus healthy controls. SEMA7A was expressed in the liver and was increased in the course of liver fibrosis, both in mice and in humans. SEMA7A was mainly expressed in HSCs with respect to other cell types in the liver and plays a critical role in regulating fibrosis. (*Am J Pathol* 2013, 183: 820–830; <http://dx.doi.org/10.1016/j.ajpath.2013.05.030>)

Fibrosis is an important process that occurs during injury in the liver and other organs. The process of hepatic fibrogenesis is characterized by increased and altered deposition of newly generated extracellular matrix in response to injury.^{1–5} Hepatic stellate cells (HSCs), a liver-specific type of pericytes located in the subendothelial space of Disse, are the main fibrogenic precursor cells. During inflammation, transdifferentiation of HSCs into fibrogenic myofibroblasts is driven by an array of cytokines among which a critical role is played by transforming growth factor- β 1 (TGF- β).^{1,6–8}

Among the different molecules potentially involved in the TGF- β —induced fibrogenetic process, an interesting role is played by semaphorins (SEMs). SEMs are a large family of phylogenetically conserved, secreted, and membrane-bound proteins that are divided into eight classes, playing a critical role in the pathogenesis of neurological disorders

and in axon guidance.^{9,10} SEMs have also been implicated in immune response and in the regulation of organogenesis, angiogenesis, apoptosis, and neoplasia.^{9–15} Furthermore a recent study showed that TGF- β is a potent inducer of semaphorin7A (SEMA7A; alias CDw108) and of its specific receptors, plexin1C and β 1-integrin (ITGB1), indicating that SEMA7A plays a key role in the pathogenesis of TGF- β —induced lung fibrosis.¹⁶

Although the role of SEMA7A in the lung has been investigated,¹⁶ the effect of SEMA7A in the liver and its

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potential role in the fibrogenetic process are still unknown. In this study we find that SEMA7A is involved in liver fibrosis, by the interaction of this molecule with the TGF- β pathway.

Materials and Methods

Animals and Treatment

Male 6- to 8-week-old wild-type (WT) mice on a C57BL/6 background were purchased from The Jackson Laboratories (Bar Harbor, ME) and used for characterization of SEMA7A expression in normal and fibrotic liver tissue and in liver cell populations. SEMA7A knockout (KO) mice bred on a C57BL/6 background were kindly provided by Dr. Alex L. Kolodkin (Johns Hopkins University School of Medicine, Baltimore, MD). The experiments, including the use of SEMA7A KO mice, were performed with WT and KO littermates.

Liver fibrosis was induced by bile duct ligation (BDL) surgery or i.p. injection of the hepatotoxin carbon tetrachloride (CCl_4). For BDL, 10 WT and 10 KO mice were anesthetized, and after midline laparotomy the common bile duct was ligated twice with 6-0 silk sutures, and the abdomen was closed. Sham-operated mice were provided as control (four mice were used for each group). Animals were sacrificed 14 days after BDL. CCl_4 (Sigma-Aldrich, St. Louis, MO) diluted 1:4 in corn oil or vehicle (corn oil) was administered i.p. to WT and KO mice (10 mice each group) at the dose of 0.5 $\mu\text{L/g}$ of body weight three times per week for a total of 12 injections.^{17–19}

Animals received standard chow diet and water *ad libitum*. For the *in vivo* study, from each animal, liver was processed for histopathology, molecular analysis, and hydroxyproline content evaluation.

For the *in vitro* study, the livers of four mice were pooled for the isolation of the different cell fractions: a total of three different isolation procedures were repeated both for WT and for KO mice, in addition to the animal used for the *in vivo* study.

Animal experiments and cell isolation studies were performed according to the guidelines of the Ancona University Institutional Animal Care and Use Committees.

Isolation of Liver Cell Populations

Digestion of the liver was performed as previously described.¹⁹ Briefly, cells filtered from the digestion of four mouse livers were centrifuged at $45 \times g$ for 1 minute to obtain the hepatocyte fraction. The remaining nonparenchymal cell fraction was collected, washed, resuspended in 8.2% Nycodenz, and put between a bottom cushion of 14.5% Nycodenz and a top layer of buffer. After centrifugation at $1598 \times g$ for 18 minutes, HSC fraction was obtained at the interface between the top and the intermediate layers, whereas the nonparenchymal cells, mainly consisting of Kupffer cells

(KCs) and endothelial cells (ECs), were localized at the interface between the intermediate and the bottom layers. The latter fraction was divided into two parts and subjected to magnetic cell sorting with the use of anti-liver sinusoidal EC antibody and anti-CD11b antibody (Miltenyi Biotec, Auburn, CA), respectively, for ECs and KCs. The HSC fraction was further cleared of KCs and ECs by magnetic cell sorting with the respective antibodies.¹⁸ The obtained cell fractions were immediately homogenized for RNA extraction.

HSC Culture

HSCs isolated from WT and SEMA7A KO mice were plated on plastic dishes and on chamber slides and incubated with Dulbecco's modified Eagle's medium enriched with antibiotics and 10% fetal bovine serum. Culture medium was changed daily.

Quiescent (qu)HSCs were obtained after 12 hours of incubation, whereas *in vitro* activated (Ac)HSCs were obtained after 5 days of incubation.¹⁹

AcHSCs were serum-starved overnight and subsequently incubated with 1 ng/mL TGF- β (R&D Systems, Minneapolis, MN) or 25 ng/mL platelet-derived growth factor (Sigma-Aldrich) for 12 hours and 30 minutes, respectively, for mRNA and protein extraction.

Eventually, 30 minutes before incubation was performed with 1 mmol/L antibiotic bacitracin (Sigma-Aldrich) or with the following specific inhibitors: 50 $\mu\text{mol/L}$ phosphatidylinositol 3-kinase inhibitor LY294002 (Calbiochem, San Diego, CA), 50 $\mu\text{mol/L}$ mitogen-activated protein kinase inhibitor PD98059 (Calbiochem), and 10 $\mu\text{mol/L}$ SMAD2/3 inhibitor SB203580 (Calbiochem).

AcHSCs were additionally subjected to 30 J/m² UV exposure for 5 minutes to check for effects on apoptosis regulation.

Adenoviral Infection and siRNA

Adenovirus-expressing SEMA7A driven by a green fluorescent protein (GFP) promoter as well as control adenovirus without insert were generated in our laboratory; the AdEasy System method technique for the construction of the viruses has been described previously.^{20–22} Adenoviruses were expanded in human embryonic kidney 293 cells.²³ HSCs at day 3 from the isolation were infected overnight with adenoviruses at a multiplicity of infection of 500. After 12 hours, media were changed, and cells were serum-starved for 24 hours before incubations and experimental procedures.

The siRNA for ITG β 1 (GenBankTM accession NM_010578) was purchased from Dharmacon (Epsom, UK) and was performed as previously reported.²⁴ Briefly, HSCs were incubated with siRNA for nontargeting vector or ITG β 1 siRNA (10 nmol/L) for 72 hours and subsequently stimulated with TGF- β for 24 hours.

Human Liver Tissue Samples

Human liver samples were obtained from patients undergoing partial liver resection or liver biopsy at the Barcelona Hospital (Liver Unit, Hospital Clínic, Barcelona, Spain). Liver samples were collected from six patients infected with hepatitis C virus (HCV) and five alcoholic (AH) patients with different degree of liver fibrosis; a total of five control samples were obtained from liver metastasis or from liver donors not suitable for transplantation (liver parenchyma with no damage).

Livers were singularly processed for immunohistochemistry (IHC) and molecular analysis.

Immunostainings

Mice and human liver tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned (5 μ m thick). Sections were stained with Sirius Red solution (saturated picric acid containing 0.1% Direct Red 80 and 0.1% Fast Green FCF) to visualize collagen deposition.

For IHC, liver sections were incubated with the following primary monoclonal antibodies: α -smooth muscle actin (α -SMA; Dako, Carpinteria, CA), SEMA7A (Santa Cruz Biotechnology Inc., Santa Cruz, CA), CD45 (Dako), and F4/80 (eBioscience, San Diego, CA), followed by incubation with the corresponding secondary antibody. Detection of positive staining was performed by using 3,3'-diaminobenzidine reagent (Sigma-Aldrich). Images were acquired with $\times 40$ magnification, and percentage of positive area was analyzed.

For double immunofluorescence, *in vitro* quHSCs (12 hours in culture) and AchHSCs (5 days in culture) or frozen section of liver parenchyma was incubated with the primary antibodies for α -SMA (Dako), SEMA7A (Santa Cruz Biotechnology Inc.), CD31, CD45, and F4/80, followed by incubation with respective fluorescent secondary antibodies (Life Technologies, Carlsbad, CA). Images were visualized with $\times 40$ magnification by fluorescence microscopy.

For immunocytochemistry, WT and KO HSCs subjected to UV exposure were incubated with primary antibody for Caspase-3 (Cell Signaling, Danvers, MA), followed by incubation with the corresponding secondary antibody. Detection of positive staining was performed with 3,3'-diaminobenzidine reagent (Sigma-Aldrich).

Quantitative Real-Time RT-PCR

Total RNA was extracted from mice and human liver, isolated liver cell fractions, and primary cultured HSCs with the use of TRIzol Reagent (Life Technologies) and reverse-transcribed to complementary cDNA. Real-time quantitative RT-PCR (RT-qPCR) was performed with Rotor-Gene 6000 instrument (Corbett Life Science, Concorde, Australia). The relative abundance of the target genes was normalized to 18S rRNA as an internal control.

Western Blot Analysis

Electrophoresis and blotting of protein extracts from primary cultured HSCs and mice and human total liver were performed as previously described.²³ Blots were incubated overnight with the following primary antibodies: anti- α -SMA, proliferating cell nuclear antigen (Sigma-Aldrich), phospho-ERK, phospho-AKT, phospho-SMAD2/3, SEMA7A (Santa Cruz Biotechnology Inc.), or Caspase-3 (Cell Signaling). After incubation with the corresponding secondary horseradish peroxidase-conjugated antibodies, blots were visualized by the enhanced chemiluminescence light method (Amersham Biosciences, Piscataway, NJ). Blots were reprobated with anti- β -actin mouse antibody (Sigma-Aldrich) to show equal loading.

Measurement of Hepatic Hydroxyproline Content

Hydroxyproline content was measured as previously described.^{25,26} Briefly, mice livers were homogenized and precipitated by trichloroacetic acid and incubated for 24 hours at 110°C in 6N HCl. After hydrolysis, samples were neutralized with 10N NaOH, oxidized with chloramine-T, and incubated in Ehrlich's perchloric acid solution at 65°C for 20 minutes. Absorbance was measured at a wavelength of 560 nm.

Statistical Methods

Results are expressed as means $\pm$ SD. Results were analyzed with the statistical method analysis of variance test. A *P* value < 0.05 was considered statistically significant.

Results

SEMA7A Is Expressed in the Liver and Increases During Injury

SEMA7A mRNA expression was assessed in liver tissue (Figure 1). By PCR analysis we observed a significant expression of SEMA7A in total mouse liver tissue, at a similar level as in the nervous system, used as positive control (Figure 1A). Moreover, SEMA7A mRNA expression increased in the course of liver injury; by RT-qPCR we observed that damaged livers of mice undergoing BDL surgery or CCl₄ injection showed a significant increase in SEMA7A mRNA expression compared with control healthy livers (Figure 1B). Fibrosis in the liver of BDL- or CCl₄-treated mice was confirmed by the increase in collagen $\alpha 1(I)$ mRNA expression compared with control livers.

SEMA7A Expression in Liver Cell Populations

We next evaluated SEMA7A mRNA expression in hepatic cell types in the course of liver fibrosis (Figure 1D). We isolated different liver cell populations by the fractionation

Figure 1 SEMA7A is expressed in the liver and increases during injury. **A:** Mouse brain and liver tissues were evaluated for SEMA7A mRNA expression by PCR. **B:** Liver specimens from control mice, or mice 2 weeks after BDL, or mice treated with 12 injections of CCl₄ were used for RNA extraction; hepatic mRNA levels of SEMA7A and collagen α1(I) were evaluated by RT-qPCR. Data were obtained from three independent experiments and represent means ± SD. * $P < 0.05$ versus control (CTRL). **C:** Purity of cells isolated from CCl₄-treated mice was evaluated by mRNA analysis of specific cell markers: HSCs, ECs, KCs, and hepatocytes (Heps) were evaluated, respectively, with α-SMA, CD31, CD68, and albumin. **D:** mRNA expression for SEMA7A was evaluated by the RT-qPCR method in different cell populations isolated from the liver of control mice and mice subjected to 12 injections of CCl₄ or 2 weeks after BDL: HSCs, ECs, KCs, and Heps. Data were obtained from three independent experiments and represent means ± SD. [†] $P < 0.05$ versus CTRL HSCs; [‡] $P < 0.05$ versus CTRL KCs. **E:** IHC for SEMA7A and double immunofluorescence staining for α-SMA and SEMA7A performed in tissue samples of fibrotic livers. MW, molecular weight.

method previously described from control mice and from mice with damaged liver, after CCl₄ or BDL treatment, and we checked for purity of each cell population by performing cell-specific marker expression by real-time RT-qPCR, as shown in Figure 1C and in Supplemental Figure S1. RT-qPCR showed that SEMA7A mRNA was mainly expressed in HSCs. Notably, SEMA7A mRNA expression was significantly higher in AchHSCs isolated from CCl₄- or BDL-treated mice than in control HSCs, with an increase of approximately threefold to fourfold ($P < 0.05$) compared with control livers. SEMA7A mRNA expression in KCs appeared to be significantly lower than in HSCs, even though a significant increased expression (1.5-fold to twofold; $P < 0.05$) was observed in cells isolated from fibrotic livers compared with control livers. Very low expression of SEMA7A with no significant differences between the cells derived from fibrotic and healthy livers was observed in ECs and hepatocytes (Figure 1C). These results are confirmed by IHC for SEMA7A performed in tissue samples of fibrotic liver, in both mice and humans, which showed positive staining in the course of liver damage with some of the positive cells depicting typical myofibroblastic-like shape cells (Figure 1E). This result was confirmed by double immunofluorescence staining that showed colocalization of SEMA7A and α-SMA protein expression in the liver of CCl₄-treated mice (Figure 1E).

SEMA7A Expression and Role in HSCs

We performed HSC isolation from healthy mice and cultured them on plastic dishes for 12 hours to obtain quHSCs and for 5 days to obtain *in vitro* AchHSCs. *In vivo* AchHSCs were obtained by isolating HSCs from mice subjected to BDL or CCl₄. As shown in Figure 2A, RT-qPCR showed a 3.5-fold increase of SEMA7A mRNA expression in AchHSCs compared with quHSCs. Similarly, the mRNA for the ITGB1 receptor subunit, one of the specific SEMA7A receptors, showed significant increased expression in AchHSCs compared with quHSCs. No significant differences for both SEMA7A and ITGB1 were observed between *in vitro* and *in vivo* activation. Plexin1C mRNA expression, the other specific receptor of SEMA7A, was conversely down-regulated during the activation status of HSCs.

Furthermore, to additionally confirm SEMA7A expression in HSCs, we performed double immunofluorescence for SEMA7A and for the marker of HSC activation α-SMA (Figure 2, B and C). Double immunofluorescence in quHSCs showed a minimal positivity for SEMA7A (red fluorescence) in the total number of cells, predominantly localized in the nucleus. As expected, no positive staining for α-SMA was observed in the field (Figure 2B). Conversely, in AchHSCs we observed a clear double-positive staining for both SEMA7A and α-SMA (green fluorescence), suggesting the

Figure 2 The expression of SEMA7A is significantly increased in *in vitro* AcHSCs versus quHSCs. **A:** mRNA was extracted from quHSCs and *in vitro* (5 days in culture) and *in vivo* (BDL- or CCL₄-treated) AcHSCs and subsequently evaluated for the expression of SEMA7A, ITGβ1, and plexin1C (Plx1C). Data were obtained from three independent experiments and represent means ± SD. **P* < 0.05 versus quHSCs. **B** and **C:** Double immunofluorescence staining for the marker of HSC activation α-SMA (green fluorescence) and SEMA7A (red fluorescence) was performed in *in vitro*-cultured quHSCs and AcHSCs.

colocalization of the two molecules. Interestingly, the SEMA7A distribution is mainly characterized by cytoplasmic localization, suggesting a potential nuclear translocation related to the molecule (Figure 2C).

AcHSCs isolated from WT and SEMA7A KO mice were incubated with the proliferation agonist platelet-derived growth factor or with apoptotic stimulus by exposing serum-starved HSCs for 12 hours to 5 minutes of 30 J/m²

Figure 3 SEMA7A does not interfere with proliferation and apoptosis signaling. **A:** Proliferating cell nuclear antigen (PCNA) protein expression and Ki-67 mRNA in an adenovirus vector overexpressing green fluorescent protein (AdGFP)- or an AdSEMA7A-infected WT and KO HSCs treated with platelet-derived growth factor (PDGF). **B:** Western blot analysis for noncleaved Caspase-3 protein expression in AdGFP- or AdSEMA7A-infected WT and KO HSCs subjected to UV exposure. Data were obtained from three independent experiments and represent means ± SD. **P* < 0.05 versus control (CTRL) HSCs. **C:** Caspase-3 IHC in WT and KO HSCs after UV exposure.

Figure 4 SEMA7A overexpression in HSCs enhances pro-fibrogenic and inflammation markers expression. HSCs were infected at the multiplicity of infection of 500 with empty vector (AdGFP) or with AdSEMA7A, stimulated with TGF- β , and eventually preincubated with bacitracin or ITGB1 siRNA. **A** and **B**: mRNA was extracted from HSCs and subsequently analyzed for the expression of ITGB1, collagen α 1(I), α -SMA, TGF- β , IL-6, tissue inhibitor of metalloproteinase (TIMP)-1, and monocyte chemoattractant protein (MCP)-1. **C**: HSCs were treated with ITGB1-specific siRNA (NT, nontargeting vector) and incubated with TGF- β . mRNA was extracted and analyzed for collagen α 1(I) and TIMP-1 expression. Data were obtained from three independent experiments and represent means $\pm$ SD; * P < 0.05 versus AdGFP; † P < 0.05 versus AdGFP + TGF- β ; ‡ P < 0.05 versus NT; § P < 0.05 versus NT + TGF- β . **D**: Western blot analysis for SEMA7A, pAKT, pERK, and pSMAD2/3 in HSCs infected with AdSEMA7A in comparison to HSCs infected with empty vector and eventually stimulated with TGF- β . Bac, bacitracin; β -actin.

UV exposure. Western blot analysis for proliferating cell nuclear antigen and RT-qPCR for Ki-67 mRNA expression as markers of proliferation showed no differences in proliferative response to platelet-derived growth factor in WT, WT overinfected with an adenovirus vector overexpressing SEMA7A (AdSEMA7A), and SEMA7A KO HSCs (Figure 3A). These data suggest the absence of any involvement of SEMA7A in the process that regulates proliferation. Furthermore, Western blot analysis for the noncleaved form of Caspase-3 as marker of apoptosis showed similar reduction after UV exposure both in WT, WT overinfected with AdSEMA7A, and KO HSCs, indicating no effect on apoptosis regulation exerted by SEMA7A in HSCs (Figure 3B). This result was confirmed by Caspase-3 immunocytochemistry, which showed an increased number of apoptotic cells both in WT and KO HSCs after UV exposure (Figure 3C).

SEMA7A Overexpression in HSCs Enhances Expression of Pro-Fibrogenic and Inflammation Markers

SEMA7A is induced by TGF- β , as indicated in the lung fibrosis model.¹⁶ To verify the biological effect of SEMA7A in HSCs, we generated AdSEMA7A on a GFP promoter. HSCs at day 3 of culture were infected overnight with the

AdSEMA7A or with an empty vector that expressed GFP (AdGFP) at a multiplicity of infection of 500. As depicted in Figure 4A, TGF- β -incubated HSCs show an increased mRNA expression of the SEMA7A receptor subunit ITGB1, which is even enhanced in HSCs infected by AdSEMA7A independently from TGF- β . AdSEMA7A also induces markers of fibrogenesis and HSC activation (collagen α 1(I), α -SMA, TGF- β), as well as markers of inflammation [IL-6, tissue inhibitor of metalloproteinase (TIMP)-1, monocyte chemoattractant protein (MCP)-1]. Specifically, endogenous TGF- β expression measured by RT-qPCR is significantly higher than in control in both TGF- β -incubated HSCs and in HSCs overexpressing SEMA7A, suggesting a potential feed-back loop related to SEMA7A activity.

Furthermore, 30 minutes before incubation of HSCs with the antibiotic bacitracin significantly reduces the TGF- β or the SEMA7A overexpression effect on HSCs. Specifically, reduction in mRNA levels of collagen α 1(I), TIMP-1, and MCP-1 are observed (data not shown) (Figure 4B).

Similar results were obtained by down-regulation of ITGB1 by specific siRNA in TGF- β -incubated HSCs (Figure 4C).

Moreover, SEMA7A protein expression was increased in TGF- β -incubated HSCs and, as expected, in AdSEMA7A-infected HSCs (Figure 4D). HSCs showed a significant

increase also in AKT and ERK phosphorylation not only in TGF- β -treated cells but also in AdSEMA7A-infected cells. Notably, SMAD2/3 phosphorylation was significantly increased in TGF- β -incubated HSCs but not in AdSEMA7A-infected HSCs (Figure 4D), suggesting that SEMA7A is not involved in SMAD2/3 phosphorylation.

TGF- β Promotes HSC Activation in a SEMA7A-Dependent Manner Through an AKT-ERK-Dependent, SMAD2/3-Independent Mechanism

AchHSCs cultured on plastic dishes for 5 days showed a comparable activation state between WT and KO mice, as indicated by the morphology and the typical myofibroblastic-like phenotype of both WT and SEMA7A KO HSCs (Figure 5B). Whether SEMA7A activity is regulated by TGF- β signaling in the liver is still unknown. In Figure 5A, RT-qPCR performed in HSCs isolated from WT mice demonstrates that SEMA7A mRNA expression was significantly higher in TGF- β -stimulated HSCs than in control HSCs, whereas no expression was detectable in HSCs isolated from SEMA7A KO mice. AchHSCs were incubated with TGF- β for 12 hours

and 30 minutes, respectively, for mRNA and protein extraction. As indicated in Figure 5A, TGF- β induced its own mRNA in both WT and KO HSCs, but both basal and TGF- β -stimulated TGF- β expression was lower in the KO HSCs than in the WT; similar results were observed also in HSCs stimulated with exogenous TGF- β . Moreover, we observed a significant reduction of fibrogenic markers collagen $\alpha 1(I)$ and connective tissue growth factor and inflammation markers (MCP-1, IL-6) in TGF- β -stimulated SEMA7A KO HSCs than in WT HSCs (Figure 5A). In addition, HSC activation, evaluated by TIMP-1 and α -SMA mRNA expression, was reduced in TGF- β -incubated SEMA7A KO HSCs compared with WT HSCs. Specifically, the increase of collagen $\alpha 1(I)$, TIMP-1, and IL-6 induced by TGF- β in WT HSCs is significantly reduced by the specific mitogen-activated protein kinase and phosphatidylinositol 3-kinase inhibitors, whereas the use of SMAD2/3 inhibitor only partially reduced the fibrogenic response (Figure 5C). Furthermore, SB203580 did not exert any effect in AdSEMA7A-infected HSCs, suggesting that SEMA7A activity is independent of SMAD pathway.

In addition, experiments were performed to define the intracellular signaling pathways involved in the process of SEMA7A-dependent fibrogenesis (Figure 5D). Western blot

Figure 5 TGF- β promotes HSC activation in an SEMA7A-dependent manner. **A:** mRNA expression for SEMA7A, TGF- β , collagen $\alpha 1(I)$, TIMP-1, α -SMA, MCP-1, connective tissue growth factor (CTGF), and IL-6 of AchHSCs isolated from WT and SEMA7A KO mice and incubated with TGF- β was evaluated by RT-qPCR. Data were obtained from three independent experiments and represent means $\pm$ SD. * $P < 0.05$ versus WT; [†] $P < 0.05$ versus KO + TGF- β ; [‡] $P < 0.05$ versus KO. **B:** *In vitro* AchHSCs isolated from both WT and SEMA7A KO mice. **C:** Collagen $\alpha 1(I)$, TIMP-1, and IL-6 were evaluated by RT-qPCR in WT HSCs infected with AdSEMA7A or incubated with TGF- β in the presence or absence of the SMAD2/3 inhibitor SB203580 (SB), the mitogen-activated protein kinase inhibitor PD98059 (PD), or the phosphatidylinositol 3-kinase inhibitor LY294002 (Ly). Data were obtained from three independent experiments and represent means $\pm$ SD. * $P < 0.05$ versus CTRL; [†] $P < 0.05$ versus control (CTRL) + TGF- β ; [‡] $P < 0.05$ versus AdSEMA7A. **D:** AKT, ERK, and SMAD2/3 protein phosphorylation was evaluated by Western blot analysis in AchHSCs isolated from WT and SEMA7A KO mice and incubated with TGF- β in the presence or absence of the inhibitors LY294002 and PD98059. β -actin.

analysis showed that AKT and ERK phosphorylation were increased in TGF- β -incubated WT HSCs and inhibited by their specific inhibitors, respectively, LY294002 and PD98059. TGF- β also induced SMAD2/3 phosphorylation. Conversely, TGF- β -incubated SEMA7A KO HSCs showed SMAD2/3 but not AKT and ERK phosphorylation (Figure 5D).

SEMA7A KO Mice Show Reduced Fibrosis Compared with WT Mice in the Course of Liver Injury

To investigate the role of SEMA7A in liver fibrogenesis *in vivo*, we induced liver injury by i.p. injection of CCl₄ both in WT and in SEMA7A KO mice. Collagen deposition, assessed by Sirius Red staining (Figure 6A), and HSC activation, assessed by IHC for α -SMA (Figure 6B), were significantly reduced in CCl₄-treated SEMA7A KO compared with CCl₄-treated WT mice. Computerized analysis indicated 16% and 21% of positive parenchyma in CCl₄-treated WT mice compared with 10% and 8.5% of positive parenchyma in CCl₄-treated SEMA7A KO mice, respectively, for Sirius Red and α -SMA immunostaining.

Consistent with these findings, liver hydroxyproline content was significantly lower in CCl₄-treated SEMA7A KO mice than in CCl₄-treated WT mice (Figure 6C). No differences were observed between WT and KO mice in the control groups.

HSC activation and collagen deposition were also evaluated by Western blot analysis and RT-qPCR. α -SMA protein expression was significantly reduced in the liver of CCl₄-treated SEMA7A KO mice compared with CCl₄-treated WT mice (Figure 6D). Similarly, mRNA expression of collagen α 1(I), α -SMA, and connective tissue growth factor were reduced in CCl₄-treated SEMA7A KO mice (Figure 6E), demonstrating the *in vivo* role of SEMA7A in the process of liver fibrogenesis.

Similar results, with a significant reduction in the level of fibrosis and liver damage were observed in BDL-treated mice as shown in Supplemental Figure S2.

Taking into consideration the regulatory effect of SEMA7A on inflammatory response, IHC for CD45 as marker of cell infiltrate and for F4/80 as marker of murine macrophage populations showed, after CCl₄ and BDL treatment (data not shown), higher positivity in WT than in SEMA7A KO mice,

Figure 6 SEMA7A KO mice show reduced fibrosis compared with WT mice after CCl₄ i.p. injection. Sirius Red histochemistry (A) and α -SMA IHC (B) were performed in paraffin liver sections of WT and SEMA7A KO mice subjected to i.p. CCl₄ or vehicle. C: Hydroxyproline content was measured in the same groups of mice, and results were expressed as micrograms of hydroxyproline per gram of liver. D: Western blot analysis for α -SMA protein expression was evaluated in WT and SEMA7A KO mice subjected to vehicle or CCl₄ treatment. E: Collagen α 1(I), α -SMA, and connective tissue growth factor (CTGF) mRNA expression were evaluated by RT-qPCR. Data represent means $\pm$ SD. * P < 0.05 versus WT; $^{\dagger}P$ < 0.05 versus KO + CCl₄; $^{\ddagger}P$ < 0.05 versus KO. CTRL, control.

Figure 7 SEMA7A exerts a potential effect in the regulation of the inflammatory response. **A:** CD45 and F4/80 IHC were performed in paraffin liver sections of WT and SEMA7A KO mice subjected to i.p. injection of CCl₄ or vehicle. **B and C:** CD45, CD68, MCP-1, and IL-6 mRNA expression were evaluated by RT-qPCR. Data represent means $\pm$ SD. * $P < 0.05$ versus control (CTRL); † $P < 0.05$ versus WT + CCl₄; ‡ $P < 0.05$ versus WT + BDL. **D:** Double immunofluorescence staining CD45-F4/80 and CD45-CD31 in CCl₄-treated WT mouse liver.

suggesting that SEMA7A plays a key role in the process of liver inflammation (Figure 7A). Furthermore, double immunofluorescence for CD45 and F4/80 as marker of KCs, and for CD45 and CD31 as marker of ECs, were performed to clarify the cell type related to the CD45 positivity; as shown in Figure 7D clear colocalization existed between CD45 and F4/80, but not between CD45 and CD31, suggesting that KCs and not ECs express CD45.

In addition, we also evaluated the expression of CD45 and CD68 (marker of KCs); mRNA expression was significantly lower in SEMA7A KO mice than in WT mice, both after CCl₄ and BDL (Figure 7B). Similarly, we found that the increase in the mRNA expression of the inflammation markers and cytokines MCP-1 and IL-6 observed in the liver of WT mice was significantly reduced in SEMA7A KO mice (Figure 7C).

SEMA7A Expression Is Increased in Human Samples of Patients with Different Levels of Liver Fibrosis

To evaluate SEMA7A potential effect, we performed experiments in liver samples obtained from patients with HCV- and AH-related fibrosis. We observed that SEMA7A was increased in the liver of patients with chronic liver diseases as assessed by RT-qPCR analysis (Figure 8A). Specifically, we observed that the livers of patients with HCV-related liver injury showed the highest levels of mRNA expression of SEMA7A compared with healthy liver

samples. We also performed Western blot analysis to evaluate SEMA7A protein expression in the same livers. Notably, we observed an increased expression of SEMA7A in both liver samples from HCV and AH compared with control healthy livers (Figure 8B).

Discussion

Liver fibrosis results from an excessive accumulation of extracellular matrix proteins occurring in all forms of chronic liver diseases.^{8,27–29} Activated HSCs and portal fibroblasts, as the main source of collagen-producing cells,^{19,30,31} are activated by fibrogenic cytokines such as TGF- β , angiotensin II, leptin, and so forth.^{17,23,32–34}

Figure 8 SEMA7A expression is increased in human samples of patients with different levels of liver fibrosis. **A:** SEMA7A mRNA expression was evaluated by RT-qPCR in the liver tissue of patients with hepatitis C virus (HCV) and alcohol (AH)-related fibrosis compared with healthy liver samples. Data represent means $\pm$ SD. * $P < 0.05$ versus control (CTRL). **B:** Western blot analysis for SEMA7A protein expression in the liver tissue of patients with liver HCV- and AH-related fibrosis compared with healthy liver samples. β ACT, β -actin.

Our study evaluated the expression and the functional role of SEMA7A in the regulation of the TGF- β —mediated liver fibrogenesis. The increased expression of SEMA7A in the course of liver injury both in mice and in human samples suggested the potential involvement of this molecule in liver fibrogenesis.^{35,36} Moreover, the cell population that indicated the highest difference in the SEMA7A expression levels between control and fibrotic liver was represented by HSCs. We therefore focused our attention on this cell population. Our experiments certainly confirm the contribution of SEMA7A in regulating TGF- β —mediated fibrogenic response in HSCs. First, the local redistribution of SEMA7A expression from the nucleus to the cytoplasm in the course of HSC activation may represent a critical point in the potential interaction between SEMA7A and TGF- β . The importance of SEMA7A is indicated primarily by the blunted effect of TGF- β in SEMA7A KO HSCs: TGF- β —incubated SEMA7A KO HSCs show a reduced expression of fibrogenic and inflammation markers. This pattern of expression was completely reverted and even enhanced in HSCs overexpressing SEMA7A. Overexpression of SEMA7A induced markers of liver fibrogenesis and HSC activation. Furthermore, as indicated by the evaluation of the endogenous expression of TGF- β , SEMA7A was able to induce the transcription of endogenous TGF- β that may lead to a positive feedback loop between SEMA7A and the fibrogenic process. This issue was supported by the reduced expression of endogenous TGF- β expression observed in SEMA7A KO HSCs. Thus, our experiments found that SEMA7A and its specific receptor ITG β 1 subunit act downstream of TGF- β signaling, participating in the process of liver fibrogenesis. Interestingly, we noticed that TGF- β —incubated SEMA7A KO HSCs show reduced phosphorylation of ERK and AKT signaling but not of SMAD2/3, suggesting the partial involvement of SEMA7A in TGF- β signaling. Notably, SMAD2/3 represents the main pathway regulating TGF- β signaling and fibrosis; in our experiments we found the possibility for TGF- β to signal in the liver through a SEMA7A-dependent SMAD2/3-independent mechanism. This issue could also explain the reason why SMAD3 KO HSCs, after *in vitro* and *in vivo* activation, show similar levels of α -SMA mRNA expression (marker of fibrogenesis) compared with WT HSCs, suggesting a noncomplete blockage of the TGF- β pathway through the SMAD signaling deletion.^{37,38}

Among the liver cell populations, as found by our experiments, SEMA7A is not solely expressed in HSCs but also in KCs, and its expression, even if to a lesser extent, is up-regulated during the liver damage, suggesting an additional fibrogenic role for SEMA7A expressed in KCs. This interesting issue will require additional studies to define specific contribution of SEMA7A in the two cell populations during the liver fibrogenetic process.

Because SEMA7A activity is strictly related to the ITG β 1 receptor, the interaction of SEMA7A-positive cells with other cell types expressing integrin receptors could be important and need to be investigated by additional studies. This aspect could indicate migration activity and cell-to-cell

interaction function that is mediated by SEMA7A in the liver.

In addition to our *in vitro* experiments, the critical role of SEMA7A in liver fibrosis was also confirmed *in vivo*: livers from SEMA7A KO mice showed reduced fibrosis and inflammation after both CCl₄ and BDL treatment compared with controls. Interestingly, the CD45 positivity in the liver of WT mice subjected to liver injury mainly colocalized with F4/80, but not with CD31, suggesting macrophage involvement in the inflammation processes associated to SEMA7A. The *in vivo* study also confirms a potential effect of SEMA7A in the regulation of the inflammatory response, as largely reported in literature, which may take an active part in the process of liver fibrogenesis, suggesting a dual fibrogenic effect mediated by SEMA7A by directly acting on HSC activation process and indirectly acting and regulating the inflammation process occurring in the course of liver damage.

In summary, our study found a new role for SEMA7A in liver fibrogenesis, and the involvement of this molecule in the TGF- β signaling in HSCs, both in mice and in humans. SEMA7A, expressed in HSCs, provides the existence of a SMAD-independent TGF- β pathway that plays an active role in liver fibrogenesis. Additional studies on SEMA7A and the liver would help to better understand the pathological mechanisms underlying fibrosis and could also provide new potential therapeutic targets for the future.

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Supplemental Data

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