Osteopontin, an Oxidant Stress Sensitive Cytokine, Up-regulates Collagen-I via Integrin $\alpha_V\beta_3$ Engagement and PI3K/pAkt/NFκB Signaling

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A key feature in the pathogenesis of liver fibrosis is fibrillar Collagen-I deposition; yet, mediators that could be key therapeutic targets remain elusive. We hypothesized that osteopontin (OPN), an extracellular matrix (ECM) cytokine expressed in hepatic stellate cells (HSCs), could drive fibrogenesis by modulating the HSC pro-fibrogenic phenotype and Collagen-I expression. Recombinant OPN (rOPN) up-regulated Collagen-I protein in primary HSCs in a transforming growth factor beta (TGFβ)–independent fashion, whereas it down-regulated matrix metalloprotease-13 (MMP13), thus favoring scarring. rOPN activated primary HSCs, confirmed by increased α-smooth muscle actin (αSMA) expression and enhanced their invasive and wound-healing potential. HSCs isolated from wild-type (WT) mice were more profibrogenic than those from OPN knockout ($Opn^{-/-}$) mice and infection of primary HSCs with an Ad-OPN increased Collagen-I, indicating correlation between both proteins. OPN induction of Collagen-I occurred via integrin $\alpha_v\beta_3$ engagement and activation of the phosphoinositide 3-kinase/phosphorylated Akt/nuclear factor kappa B (PI3K/pAkt/NFκB)–signaling pathway, whereas cluster of differentiation 44 (CD44) binding and mammalian target of rapamycin/70-kDa ribosomal protein S6 kinase (mTOR/p70S6K) were not involved. Neutralization of integrin $\alpha_v\beta_3$ prevented the OPN-mediated activation of the PI3K/pAkt/NFκB–signaling cascade and Collagen-I up-regulation. Likewise, inhibition of PI3K and NFκB blocked the OPN-mediated Collagen-I increase. Hepatitis C Virus (HCV) cirrhotic patients showed coinduction of Collagen-I and cleaved OPN compared to healthy individuals. Acute and chronic liver injury by CCl_4 injection or thioacetamide (TAA) treatment elevated OPN expression. Reactive oxygen species up-regulated OPN *in vitro* and *in vivo* and antioxidants prevented this effect. Transgenic mice overexpressing OPN in hepatocytes (Opn^{HEP} Tg) mice developed spontaneous liver fibrosis compared to WT mice. Last, chronic CCl_4 injection and TAA treatment caused more liver fibrosis to WT than to $Opn^{-/-}$ mice and the reverse occurred in Opn^{HEP} Tg mice. *Conclusion*: OPN emerges as a key cytokine within the ECM protein network driving the increase in Collagen-I protein contributing to scarring and liver fibrosis. (HEPATOLOGY 2012;55:594-608)

Fibrogenesis, or activation of the wound-healing response to persistent liver injury, is characterized by changes in the composition and quantity of extracellular matrix (ECM) deposits distorting the normal hepatic architecture by forming fibrotic scars. Failure to degrade accumulated ECM is a major reason why fibrosis progresses to cirrhosis. Emerging antifibrogenic therapies aim at inhibiting the activation of

Abbreviations: p70S6K, 70-kDa ribosomal protein S6 kinase; Abs, antibodies; ALT, alanine aminotransferase; BSO, L-buthionine sulfoximine; CD, cluster of differentiation; CYP2E1, cytochrome P450 2E1; ECM, extracellular matrix; GSH, glutathione; GSH-EE, glutathione ethyl ester; HCV, hepatitis C virus; H&E, hematoxylin and eosin; HSCs, hepatic stellate cells; IgG, immunoglobulin G; IHC, immunohistochemical; IKK, I kappa B kinase; MMP, matrix metalloprotease; MO, mineral oil; NFκB, nuclear factor kappa B; OPN, osteopontin; $Opn^{-/-}$*, osteopontin knockout mice;* Opn^{HEP} *Tg, transgenic mice overexpressing OPN in hepatocytes; pAkt, phosphorylated Akt; PDTC, pyrrolidine dithiocarbamate; pERK, phosphorylated extracellular signal-related kinase; PI3K, phosphoinositide 3-kinase; rOPN, recombinant OPN; pp38, phosphorylated p38; SAM, S-adenosylmethionine; SEM, standard error of the mean; αSMA, α-smooth muscle actin; TAA, thioacetamide; TGFβ, transforming growth factor beta; WT, wild type.*

profibrogenic cells to prevent fibrillar Collagen-I deposition, degrading excessive ECM to recover normal liver architecture and restoring functional liver mass.

Although different cell types contribute to the increase in fibrillar Collagen-I during hepatic fibrogenesis, they all undergo a common process of differentiation and acquisition of a classical myofibroblast-like phenotype. Hepatic stellate cells (HSCs) are considered central ECM-producing cells within the injured liver,[1] playing a significant role in Collagen-I deposition when hepatocellular injury is concentrated within the liver lobules and sinusoids. In the healthy liver, they reside in the sinusoidal space of Disse; however, during chronic injury, they activate while acquiring motile, proinflammatory and profibrogenic properties.[2] Activated HSCs migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM, mostly Collagen-I and regulating ECM remodeling. Up-regulation of fibrillar Collagen-I is thus a key event leading to scarring, the pathophysiological hallmark of liver fibrosis.

Though some current therapies have proven beneficial, dissecting key profibrogenic mechanisms, pathways and mediators of disease progression is vital. Several studies have identified osteopontin (OPN) as significantly up-regulated during liver injury and in HSCs.[3-6] OPN is a soluble cytokine and a matrix-bound protein that can remain intracellular or is secreted, hence allowing autocrine and paracrine signaling.[7,8] OPN, as a matricellular phosphoglycoprotein, functions as an adaptor and modulator of cell-matrix interactions.[8] Among its many roles, it regulates cell migration, ECM invasion and cell adhesion resulting from its ability to bind integrins—through its RGD motif—or to cluster of differentiation (CD)44—by a cryptic site (SVVYGLR)—exposed after cleavage by thrombin, plasminogen, plasmin, cathepsin B and some matrix metalloproteinases (MMPs).[5,9] OPN expression increases in tumorigenesis, angiogenesis and in response to inflammation, cellular stress and injury.[10-14] OPN plays an important role in regulating tissue remodeling and cell survival as well as in chemoattracting inflammatory cells.[15] Moreover, osteopontin knockout ($Opn^{-/-}$) mice show matrix disarrangement and alteration of collagen fibrillogenesis in cartilage, compared to their wild-type (WT) littermates.[16]

There is limited information on the contribution of OPN to the HSC profibrogenic behavior and the molecular mechanisms and signaling pathways involved in governing Collagen-I protein expression during the fibrogenic response to liver injury.[3-6,17] Because OPN is expressed in HSCs,[3-6] we hypothesized that OPN could trigger signals capable of up-regulating Collagen-I *per se*, hence acting as a feed-forward mechanism promoting scarring. Therefore, the major aim of this work was to determine how OPN could become a profibrogenic "switch" and to characterize the underlying cellular mechanism for this effect. In the present study, we identified a role for intracellular OPN in increasing Collagen-I, the HSC membrane proteins engaged by extracellular OPN, the proximal signaling molecules/stress-sensitive kinases activated upon binding that trigger the profibrogenic cascade, the ability of OPN to respond to oxidant stress, and the effect of *Opn* ablation or overexpression on Collagen-I deposition *in vivo*.

Materials and Methods

Please see Supporting Materials for a detailed description of experimental procedures.

Results

Recombinant OPN Slightly Increases HSC Proliferation Rates and Promotes HSC Migration. Recombinant OPN (rOPN) did not alter HSCs viability, but slightly induced proliferation rates, both in rat and in human HSCs (Supporting Fig. 1);

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Additional Supporting Information could be found in the online version of this article.

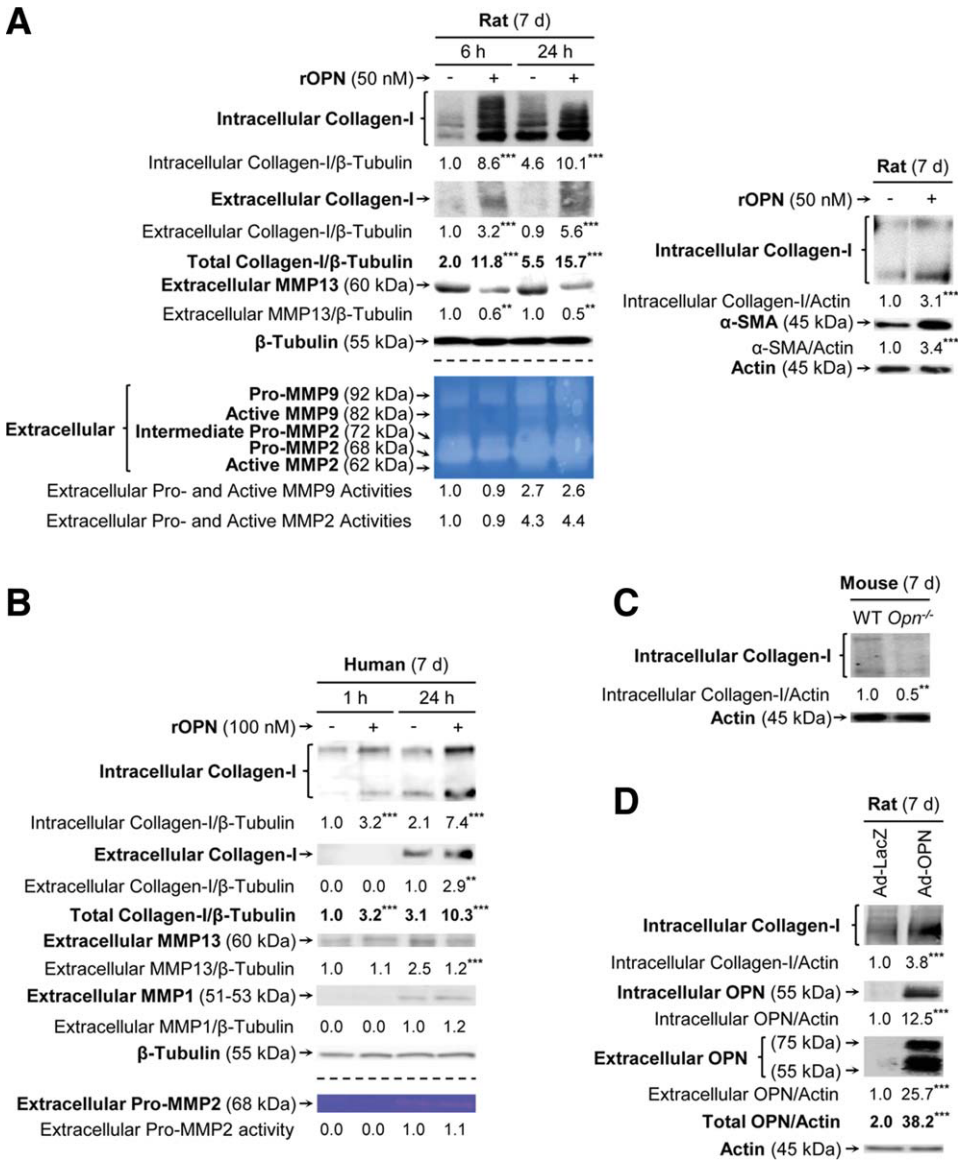

Fig. 1. Profibrogenic effects of rOPN in primary HSCs. Primary rat HSCs cultured for 7 days were treated with 0-50 nM of rOPN for 6 and 24 hours. Western blotting analysis of intra- and extracellular Collagen-I, extracellular MMP13 and β-tubulin. Gelatine zymography showing extracellular pro-, intermediate, and active MMP2 and 9 (A, left). Western blotting analysis of intracellular Collagen-I, αSMA and actin in rat HSCs treated with 0-50 nM of rOPN for 24 hours (A, right). Human HSCs cultured for 7 days were treated with 0-100 nM of rOPN for 1 and 24 hours. Western blotting analysis of intra- and extracellular Collagen-I, extracellular MMP13 and 1 and β-tubulin. Pro-MMP2 activity was measured by gelatine zymography. Extracellular MMP9 activity was undetectable (not shown) (B). Western blotting analysis of intracellular Collagen-I and actin in HSCs from WT and $Opn^{-/-}$ mice. Extracellular Collagen-I was undetectable (C). Western blotting analysis of intracellular Collagen-I and OPN, as well as extracellular OPN in rat HSCs infected with Ad-LacZ or with Ad-OPN for 48 hours. Extracellular Collagen-I was undetectable (D). Results are expressed as average values. Experiments were performed in triplicates four times. $^{**}P < 0.01$ and $^{***}P < 0.001$ for rOPN, $Opn^{-/-}$ or Ad-OPN versus control, WT or Ad-LacZ, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

however, rOPN caused a 2-fold increase in the invasive potential or chemotaxis (Supporting Fig. 2A, 2B) and enhanced the wound-closure ability of rat HSCs (Supporting Fig. 2C), important functions gained by HSC during their activation that contribute to their profibrogenic ability. Neutralizing antibodies (Abs) to $\alpha_v\beta_3$ integrin and to OPN blocked the effects on HSC invasion (not shown) and on wound closure ability (Supporting Fig. 2C).

rOPN Induces Profibrogenic Effects in Primary HSCs. Upon stimulation with rOPN, rat HSCs upregulated intra- and extracellular Collagen-I in a time-dependent fashion (Fig. 1A, left). Denatured rOPN did not elevate Collagen-I, thus confirming the specificity of the rOPN effect on Collagen-I in HSCs (not shown). rOPN lowered extracellular MMP13 protein by 50%, contributing to extracellular Collagen-I accumulation. Reciprocal modulation of MMP13 and

Collagen-I has been previously described in rat HSCs.[18] Extracellular pro-, intermediate, and active MMP2 and 9 remained unchanged (Fig. 1A, left). Likewise, tissue inhibitor of MMP1 was comparable (not shown). rOPN induced rat HSC activation, as shown by the increase in Collagen-I and alpha smooth muscle actin (αSMA) proteins (Fig. 1A, right). Analogous results were observed in human HSCs (Fig. 1B).

Because of the ability of HSCs to secrete transforming growth factor beta (TGFβ),[19] along with its well-known profibrogenic effect,[20] rat HSCs were treated with anti-TGFβ Ab. Neutralization of TGFβ did not alter the rOPN-mediated induction of Collagen-I, thus implying a mechanism independent of TGFβ production by HSCs (Supporting Fig. 3).

To dissect whether intracellular OPN could play an autocrine role in modulating Collagen-I expression, HSCs were isolated from WT and *Opn*$^{-/-}$ mice. WT HSCs appeared more profibrogenic than *Opn*$^{-/-}$ HSCs, because intracellular Collagen-I expression at 7 days of culture was higher in WT HSCs than in *Opn*$^{-/-}$ HSCs (Fig. 1C). Infection of rat HSCs with an Ad-OPN increased intracellular Collagen-I and intra- and extracellular OPN, compared to HSCs infected with Ad-LacZ (Fig. 1D). Therefore, a novel autocrine role for intracellular OPN in modulating Collagen-I deposition was identified.

Anti-$\alpha_v\beta_3$ Integrin Blocks the rOPN-Driven Collagen-I Increase. Because OPN is also a soluble cytokine and a matrix-bound protein, we next evaluated the role of extracellular OPN-mediated signaling (i.e., paracrine role) on Collagen-I induction in HSCs. OPN signals via integrins—mostly integrin $\alpha_v\beta_3$ highly expressed in HSCs[21-23]—and via CD44, also expressed in HSCs.[24] Incubation with anti-$\alpha_v\beta_3$ integrin blocked the rOPN-driven total Collagen-I (intra- plus extracellular) increase in rat HSCs, whereas no major effect was observed by anti-CD44 (Fig. 2A). Neutralization of other integrins (i.e., β_1, β_5 and β_6) failed to prevent the increase in Collagen-I by rOPN (not shown). Similar results were observed in human HSCs (not shown).

rOPN Increases Collagen-I Protein via Activation of the PI3K/pAkt/NFκB–Signaling Pathway. Given that Collagen-I protein is highly responsive to oxidant stress-sensitive kinases, we analyzed the expression of protein kinases involved in regulating Collagen-I expression, such as phosphorylated p38 (pp38),[25,26] phosphorylated extracellular signal-related kinase (pERK1/2),[27] pJNK,[28,29] phosphoinositide 3-kinase (PI3K), and phosphorylated Akt (pAkt).[26,30] Only PI3K and the ratio of pAkt ^{473}Ser/Akt were elevated time dependently by rOPN up to 3 hours in rat HSCs (Fig. 2B) and up to 1 hour in human HSCs (Supporting Fig. 4A).

Because PI3K/pAkt are upstream of I kappa B kinase (IKK) and the IKK complex is central for the activation of nuclear factor kappa B (NFκB) to regulate Collagen-I,[26,31] we focused on analyzing this signaling pathway. There was up-regulation of the ratios pIKKα,β $^{176/180}$Ser/IKKα,β, pIκBα ^{32}Ser/IκBα as well as of nuclear/cytosolic p65 in OPN-treated rat HSCs (Fig. 2C). However, involvement of the target of rapamycin/70-kDa ribosomal protein S6 kinase (mTOR-p70S6K) cascade, a translational regulatory mechanism downstream of PI3K and pAkt ^{473}Ser for regulating Collagen-I,[32] was precluded, because rOPN neither altered mTOR and p706SK expression (Fig. 2D) nor induced mTOR phosphorylation at ^{2448}Ser or ^{2481}Ser in rat HSCs (undetectable).

To further define the molecular mechanism for Collagen-I induction under rOPN challenge, we evaluated the potential role of the activation of these two stress-sensitive kinases (i.e., PI3K and pAkt) and of the NFκB-signaling pathway. Wortmannin, a PI3K inhibitor, neither altered rat HSCs viability (100% by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay), morphology nor proliferation rates (Supporting Fig. 4B and not shown); however, three different doses of wortmannin down-regulated total Collagen-I expression in rat HSCs cotreated with rOPN (Fig. 3A, top). Similar effects were observed by coincubation with LY294002, a second PI3K inhibitor (Fig. 3A, bottom), thus linking OPN, PI3K-pAkt activation and Collagen-I up-regulation in rat HSCs. Comparable results were observed in human HSCs (Supporting Fig. 4C). Last, inhibitors of pp38, pERK1/2 and pJNK signaling did not prevent the increase in Collagen-I by rOPN (not shown).

Addition of pyrrolidine dithiocarbamate (PDTC) to block NFκB signaling prevented the rOPN-driven increase in Collagen-I in rat HSCs (Fig. 3B, top). Analogous effects were observed by coincubation with CAY10512—a second inhibitor of NFκB signaling (Fig. 3B, middle). Moreover, HSCs infected with Ad-NFκB-Luc and treated with rOPN for 24 hours showed a 2-fold increase in luciferase activity, compared to non-rOPN-treated Ad-NFκB-Luc-infected cells (Fig. 3B, bottom). Both wortmannin and an $\alpha_v\beta_3$ integrin neutralizing Ab blunted the rOPN-mediated effect on the ratios pIKKα,β $^{176/180}$Ser/IKKα,β, pIκBα ^{32}Ser/IκBα as well as on nuclear/cytosolic p65 (Fig. 3C), suggesting engagement of OPN with integrin $\alpha_v\beta_3$, PI3K-pAkt activation and NFκB signaling to

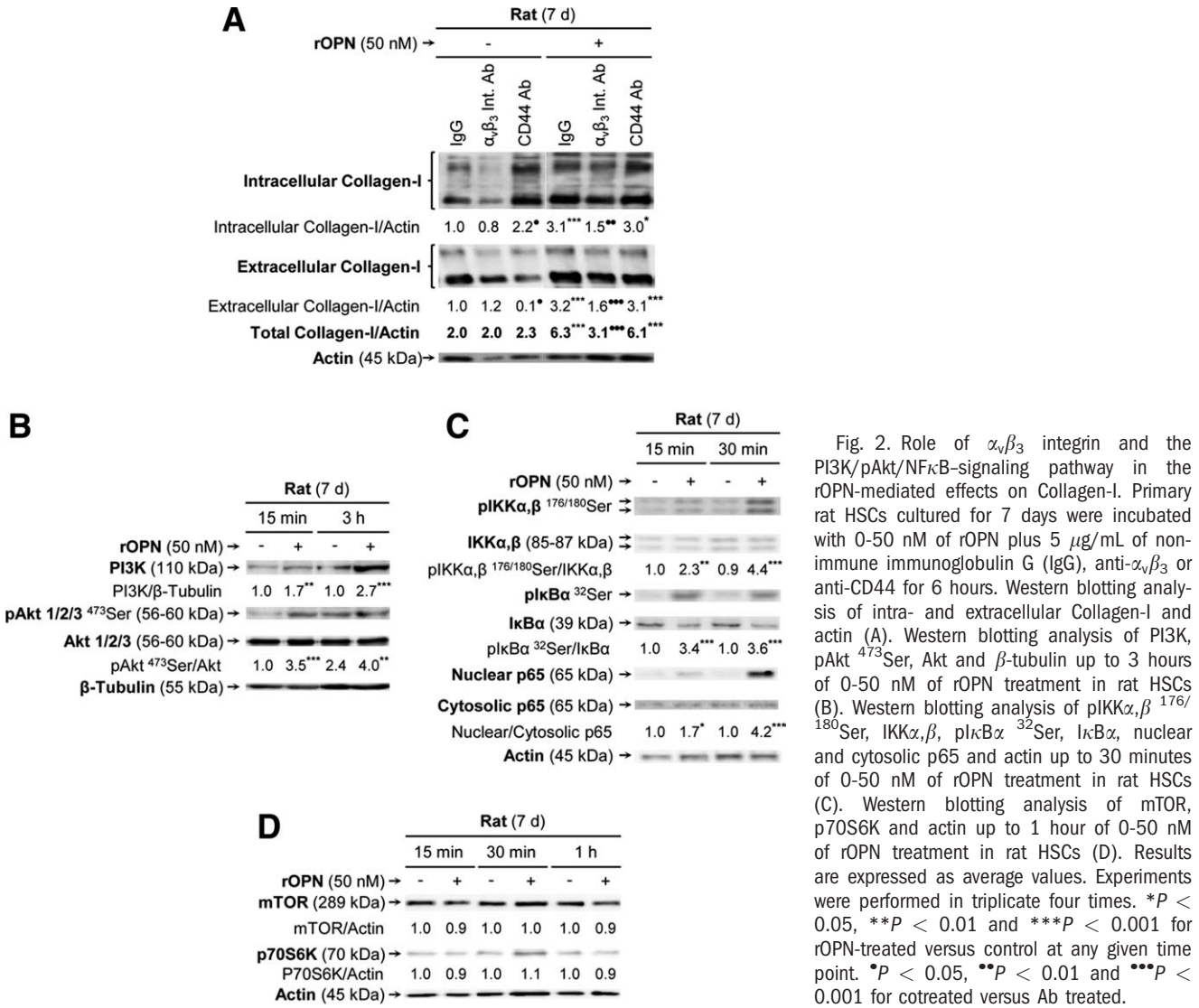

Fig. 2. Role of $\alpha_v\beta_3$ integrin and the PI3K/pAkt/NFκB–signaling pathway in the rOPN-mediated effects on Collagen-I. Primary rat HSCs cultured for 7 days were incubated with 0-50 nM of rOPN plus 5 μg/mL of non-immune immunoglobulin G (IgG), anti-$\alpha_v\beta_3$ or anti-CD44 for 6 hours. Western blotting analysis of intra- and extracellular Collagen-I and actin (A). Western blotting analysis of PI3K, pAkt ^{473}Ser, Akt and β-tubulin up to 3 hours of 0-50 nM of rOPN treatment in rat HSCs (B). Western blotting analysis of pIKKα,β $^{176/180}$Ser, IKKα,β, pIκBα ^{32}Ser, IκBα, nuclear and cytosolic p65 and actin up to 30 minutes of 0-50 nM of rOPN treatment in rat HSCs (C). Western blotting analysis of mTOR, p70S6K and actin up to 1 hour of 0-50 nM of rOPN treatment in rat HSCs (D). Results are expressed as average values. Experiments were performed in triplicate four times. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for rOPN-treated versus control at any given time point. $^\bullet P < 0.05$, $^{\bullet\bullet}P < 0.01$ and $^{\bullet\bullet\bullet}P < 0.001$ for cotreated versus Ab treated.

up-regulate Collagen-I expression in rat HSCs. Last, blocking $\alpha_v\beta_3$ integrin prevented the increase in PI3K, the ratio pAkt ^{473}Ser/Akt and Collagen-I by rOPN in rat HSCs (Fig. 3D). In summary, these results established a connection among rOPN, $\alpha_v\beta_3$ integrin, PI3K-pAkt activation and the NFκB-signaling pathway to drive Collagen-I up-regulation in rat HSCs in a paracrine manner.

OPN Expression Is Up-regulated in Liver Fibrosis. Samples from stage 3 hepatitis C virus (HCV) cirrhotic patients displayed a correlation between elevated Collagen-I and cleaved OPN protein (~55-, ~42- and ~25-kDa isoforms) compared to healthy individuals. Fully modified (glycosylated and phosphorylated) monomeric OPN, typically running at ~75 kDa, was not detectable (Fig. 4A).

To determine whether OPN also increased during liver injury in mice, we used well-established *in vivo* models to induce liver fibrosis, such as CCl_4 injection and thioacetamide (TAA) treatment.[33] These drugs undergo cytochrome P450 metabolism leading to significant oxidant stress, inflammation and hepatocyte necrosis within hours. The ~25-kDa OPN form was markedly induced in acute and chronic models of liver injury, whereas the ~55-kDa OPN form was elevated only under chronic CCl_4 injection and TAA treatment (Fig. 4B). Hence, there was an association between OPN induction, OPN proteolytic processing and the extent of liver fibrosis, both in humans and in mice.

Next, we evaluated the specific localization of the OPN induction in the liver. Nontreated livers showed OPN^+ biliary epithelial cells (not shown). Primary HSCs isolated from WT mice and cultured for 6 days were OPN^+ (Fig. 4C, left). Immunohistochemical (IHC) analysis revealed OPN expression in HSC,[30] biliary epithelial cells,[4,6,34] oval cells and, mostly, in

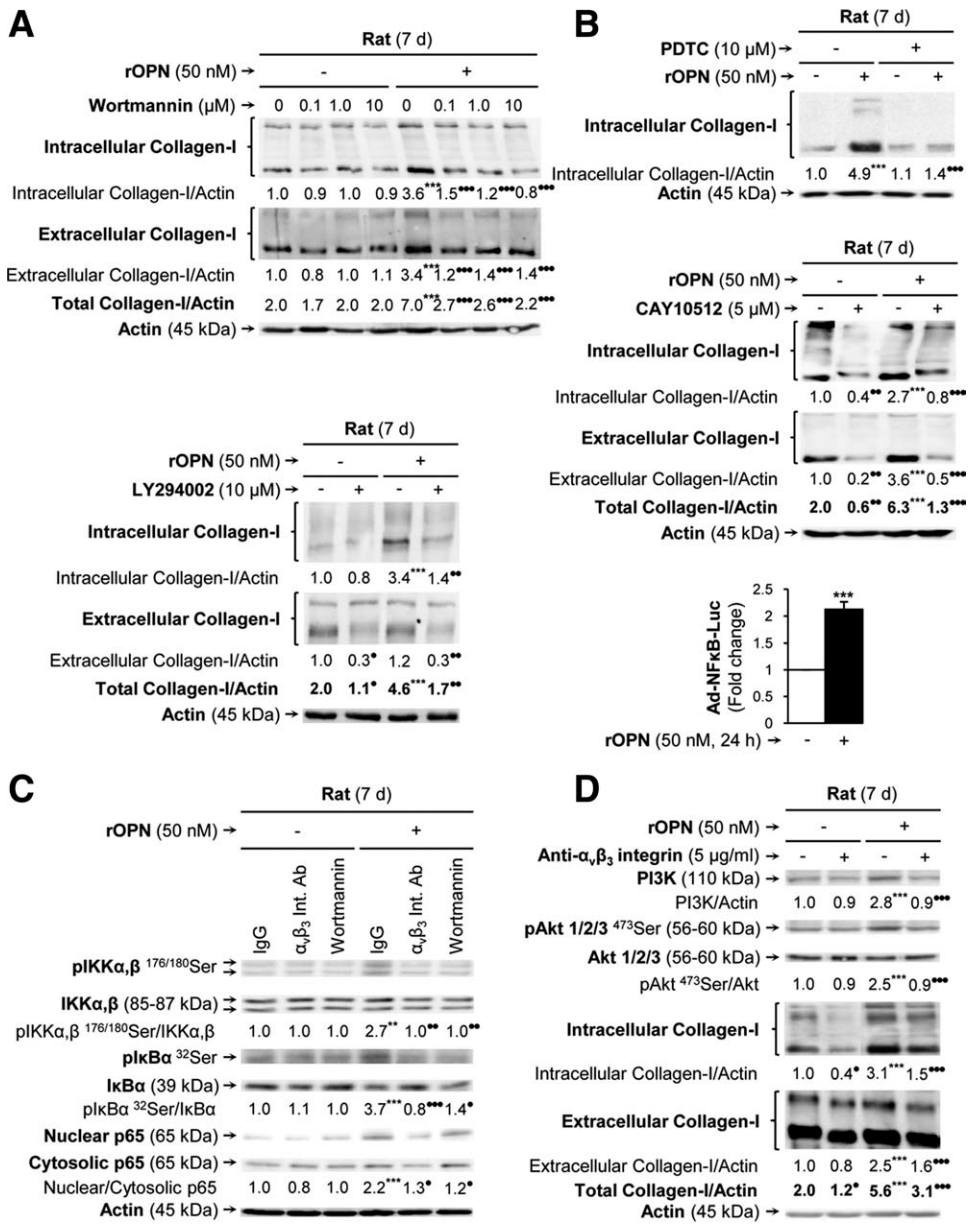

Fig. 3. Blocking $\alpha_v\beta_3$ integrin, PI3K-pAkt activation and the NFκB-signaling pathway prevents rOPN-mediated effects on Collagen-I. Primary rat HSCs cultured for 7 days were treated with 0-50 nM of rOPN or cotreated with 0-10 μM of wortmannin, 0-10 μM of LY294002, 0-10 μM of PDTC, 0-5 μM of CAY10512, or with 5 μg/mL of nonimmune IgG or a neutralizing Ab to integrin $\alpha_v\beta_3$. Western blotting analysis showing that the rOPN-mediated induction of Collagen-I in HSCs was blunted by 0.1, 1 and 10 μM of wortmannin (A, top), LY294002 (A, bottom), PDTC (B, top) and CAY10512 (B, middle). Infection of HSCs with Ad-NFκB-Luc for 48 hours and treatment with 50 nM of rOPN for 24 hours increased luciferase activity over that of nontreated Ad-NFκB-Luc–infected cells (B, bottom). Both integrin $\alpha_v\beta_3$ Ab and wortmannin blunted the rOPN-mediated induction of the ratios pIKKα,β $^{176/180}$Ser/IKKα,β, pIκBα ^{32}Ser/IκBα and nuclear/cytosolic p65 (C). Neutralizing Ab to integrin $\alpha_v\beta_3$ prevented the induction of PI3K, the ratio pAkt ^{473}Ser/Akt and Collagen-I by rOPN in HSCs (D). Results are expressed as average values. Experiments were performed in triplicate four times. $^{**}P < 0.01$ and $^{***}P < 0.001$ for rOPN-treated versus control. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ and $^{\bullet\bullet\bullet}P < 0.001$ for inhibitor or Ab cotreated versus rOPN treated or control.

damaged hepatocytes in WT mice injected with CCl_4 for 1 month (Figure 4C, middle). Similar results were observed under TAA treatment, although hepatocytes showed punctated staining (Fig. 4C, right). Insets show OPN$^+$ HSCs in both models. In the early stages of CCl_4- and TAA-mediated liver injury, Kupffer cells were also OPN$^+$ (not shown); however, the staining faded with disease progression. Of note, granular OPN$^+$ staining—typical of secreted proteins—appeared in focal-septal hepatocytes (Fig. 4C, middle). There was colocalization of OPN$^+$ staining with αSMA$^+$ (an HSCs activation marker) under TAA treatment (Fig. 4D) and by CCl_4 injection (not shown).

Because liver fibrosis is associated with significant oxidant stress, to dissect whether OPN was responsive

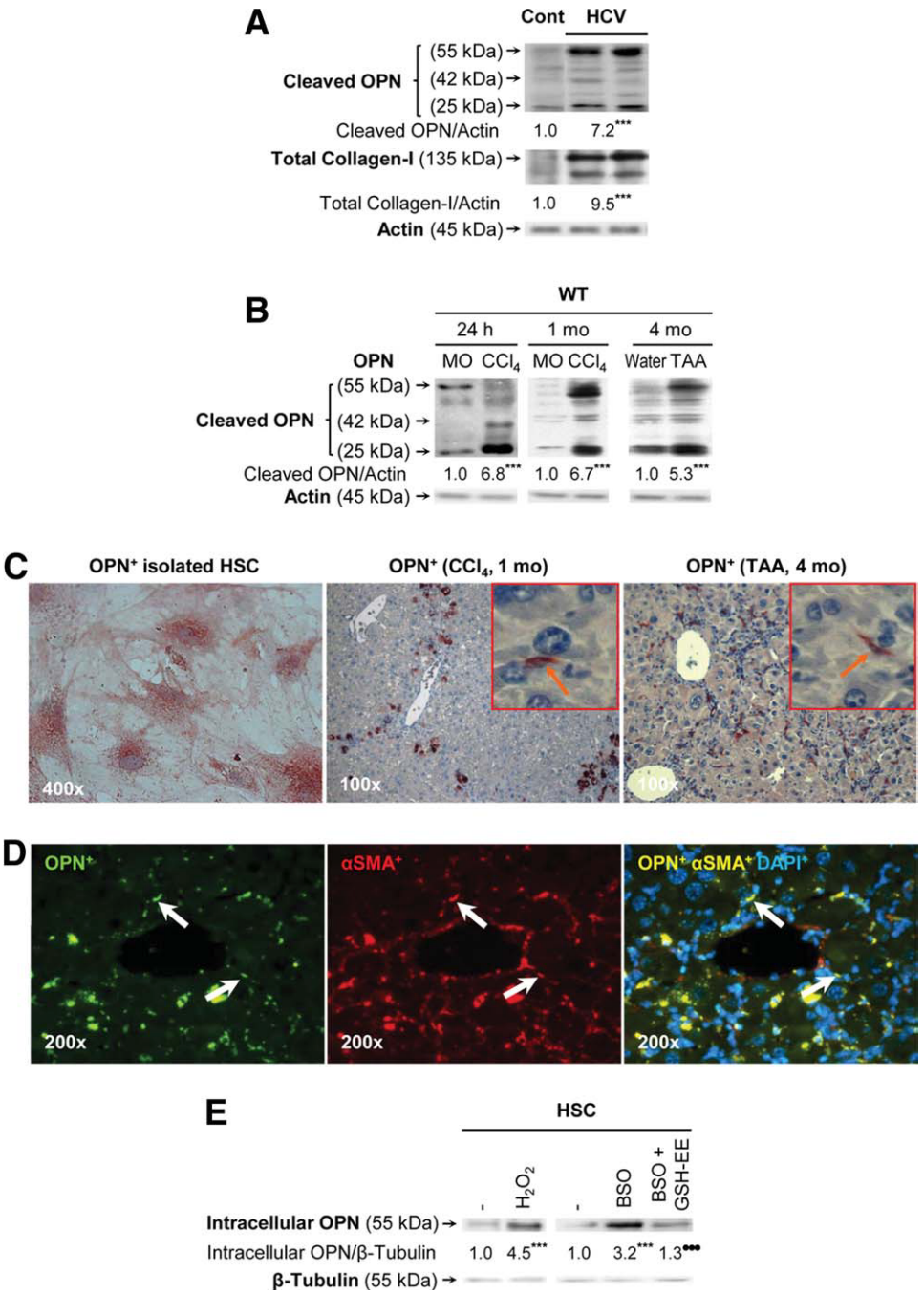

Fig. 4. OPN expression is induced during liver injury and under oxidant stress conditions. Western blotting analysis of cleaved OPN, total Collagen-I and actin in livers from control and from patients with stage 3 HCV-induced cirrhosis (A). Western blotting analysis of cleaved OPN and actin in livers of mice injected with CCl_4 for 24 hours (acute liver injury), with CCl_4 for 1 month or with TAA for 4 months (chronic liver injury) (B). In (A) and (B), fully modified (glycosylated and phosphorylated) monomeric OPN was not detected. Immunocytochemistry for OPN in primary HSCs isolated from WT mice and cultured for 6 days (C, left). IHC depicting significant OPN expression in HSCs, biliary epithelial cells and hepatocytes at 1 month of CCl_4 injection (C, middle) and in HSCs, biliary epithelial cells, oval cells and hepatocytes after 4 months of TAA treatment (C, right). Insets show OPN^+ HSCs in both models (➔). Immunofluorescence showing colocalization of OPN^+ with αSMA^+ (an HSC activation marker) after 4 months of TAA treatment (D). Western blotting analysis of intracellular OPN and β-tubulin in HSCs in the presence of two prooxidants (H_2O_2 and BSO) and an antioxidant (GSH-EE) (E). Results are expressed as average values. Experiments were performed in triplicate four times. $^{***}P < 0.001$ for HCV, CCl_4, TAA or prooxidant treated versus control, mineral oil (MO) or water, respectively. $^{•••}P < 0.001$ for BSO+GSH-EE cotreated versus BSO treated.

to reactive oxygen species, HSC were challenged with H_2O_2—a prooxidant typically generated during CCl_4 metabolism—or with L-buthionine sulfoximine (BSO), which depletes glutathione (GSH). Both treatments increased OPN expression in HSCs, whereas cotreatment with glutathione ethyl ester (GSH-EE) to

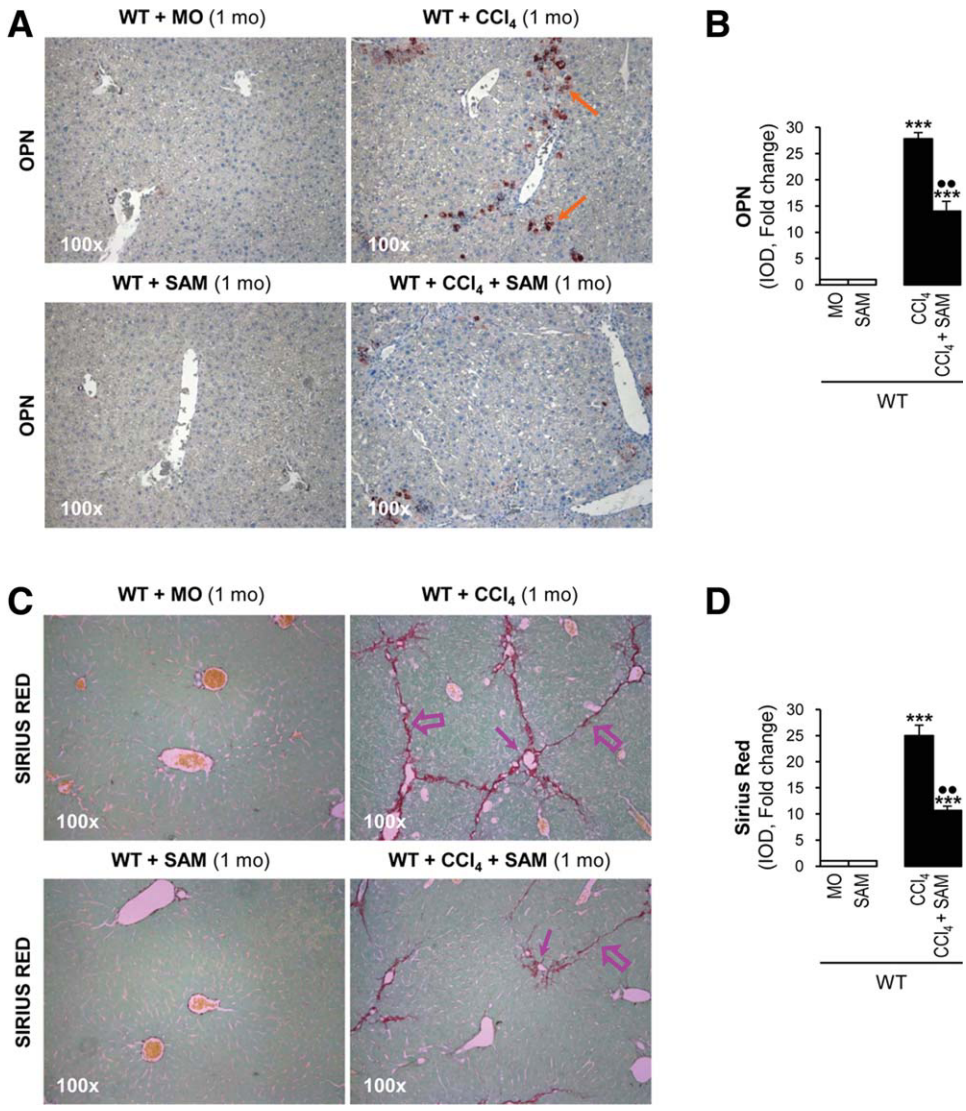

Fig. 5. SAM protects WT mice from CCl_4-induced chronic liver injury. C57BL/6J WT mice were injected MO, SAM+MO, CCl_4 or CCl_4+SAM for 1 month. Cotreated mice showed decreased OPN expression (A), which was quantified by morphometry analysis (B). Likewise, fibrosis was less apparent in cotreated mice than in CCl_4-injected mice, as shown by Sirius red/fast green staining (C) and morphometry analysis (D). Results are expressed as mean values ± standard error of the mean (SEM). $n = 8$/group; $^{***}P < 0.001$ for CCl_4 or CCl_4+SAM versus MO or SAM; $^{\bullet\bullet}P < 0.01$ for CCl_4+SAM versus CCl_4.

restore GSH levels, blunted this effect (Fig. 4E). To validate the induction of OPN by oxidant stress *in vivo*, WT mice were CCl_4 injected for 1 month in the presence or absence of *S*-adenosylmethionine (SAM), an antioxidant known to restore GSH levels. Coinjection with SAM lowered OPN protein (Fig. 5A, 5B) and the extent of liver fibrosis (Fig. 5C, 5D) by 50% when compared to mice injected with CCl_4 alone. In summary, these data proved the ability of OPN to respond to drug-induced liver injury and to oxidant stress.

WT Show More CCl_4-Induced Chronic Liver Injury and Fibrosis Than $Opn^{-/-}$ Mice. Fibrosis typically develops as a result of chronic liver injury. To decipher the role of OPN in the progression of liver disease, we tested whether chronic CCl_4 injection could lead to differences in the extent of liver fibrosis. CCl_4-injected C57BL/6J WT showed greater alanine aminotransferase (ALT) activity and more inflammation, hepatocyte-ballooning degeneration and necrosis than $Opn^{-/-}$ mice (Fig. 6A-6E). Cytochrome P450 2E1 (CYP2E1) expression was similar in WT and $Opn^{-/-}$ mice, indicating that the extent of liver injury in these mice was not the result of different CCl_4 metabolism (Fig. 6F).

In addition, CCl_4-injected WT mice presented elevated collagenous proteins, portal fibrosis, bridging fibrosis, scar thickness, Brunt fibrosis score and Sirius red and Collagen-I morphometry compared to $Opn^{-/-}$ mice (Fig. 7A-7E). The above-described results were validated in WT and $Opn^{-/-}$ 129sv mice (Supporting Figs. 5 and 6). Transgenic mice overexpressing OPN in hepatocytes (Opn^{HEP} Tg) injected with CCl_4 for 1 month showed similar ALT activity, necrosis and inflammation, but significant periportal, bridging and sinusoidal fibrosis, along with increased Collagen-I scar thickness, compared to WT mice (Fig. 8). Moreover, Opn^{HEP} Tg mice developed spontaneous perivenular, perisinusoidal and portal fibrosis over time (1 year) in the absence of any profibrogenic treatment (Supporting Fig. 7). In aggregate, the data

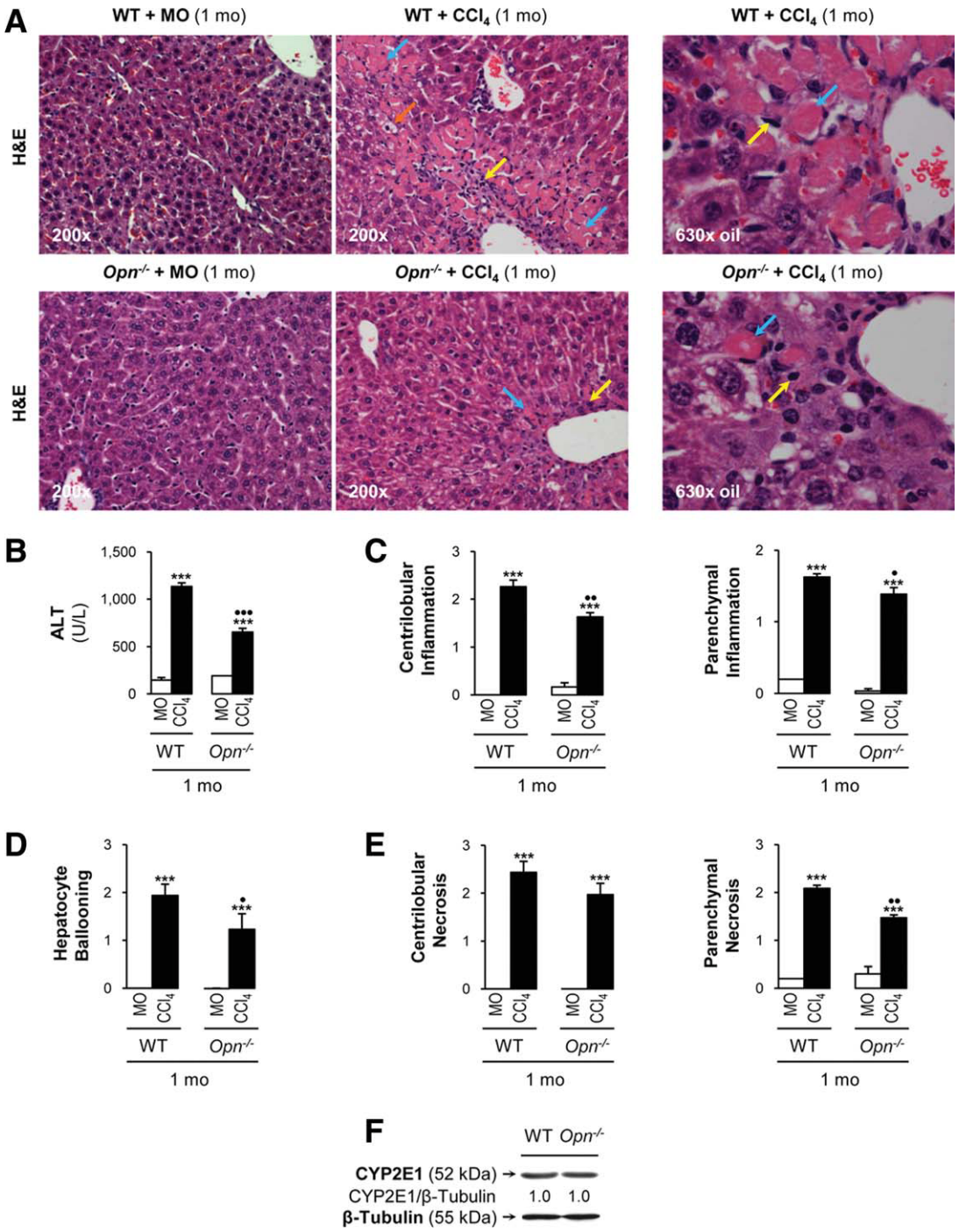

Fig. 6. WT mice show more CCl_4-induced chronic liver injury than $Opn^{-/-}$ mice. C57BL/6J WT and $Opn^{-/-}$ mice were injected with CCl_4 or MO for 1 month. Hematoxylin and eosin (H&E) staining revealed more centrilobular necrosis (➔), centrilobular inflammation (➔) and hepatocyte-ballooning degeneration (➔) in CCl_4-injected WT than in $Opn^{-/-}$ mice (A). ALT activity (B), centrilobular and parenchymal inflammation scores (C), hepatocyte-ballooning degeneration score (D), centrilobular and parenchymal necrosis scores (E). Western blotting analysis showing similar CYP2E1 expression in WT and $Opn^{-/-}$ mice (F). Results are expressed as mean values ± SEM. $n = 8$/group; $^{***}P < 0.001$ for CCl_4 versus MO; $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ and $^{\bullet\bullet\bullet}P < 0.001$ for $Opn^{-/-}$+CCl_4 versus WT+CCl_4.

suggest that OPN plays a major role in chronic CCl_4-induced hepatic fibrosis by regulating scar formation.

WT Mice Show Significant Liver Fibrosis Under Chronic TAA Treatment Compared to $\mathbf{Opn^{-/-}}$ ***Mice.*** To confirm the results obtained under chronic CCl_4 injection, we used TAA treatment as a second model of chronic drug-induced liver fibrosis. Sirius red/fast green staining and Collagen-I IHC showed stage >3 fibrosis in TAA-treated WT and ~1-2 in $Opn^{-/-}$ mice with clear induction of Collagen-I deposition in TAA-

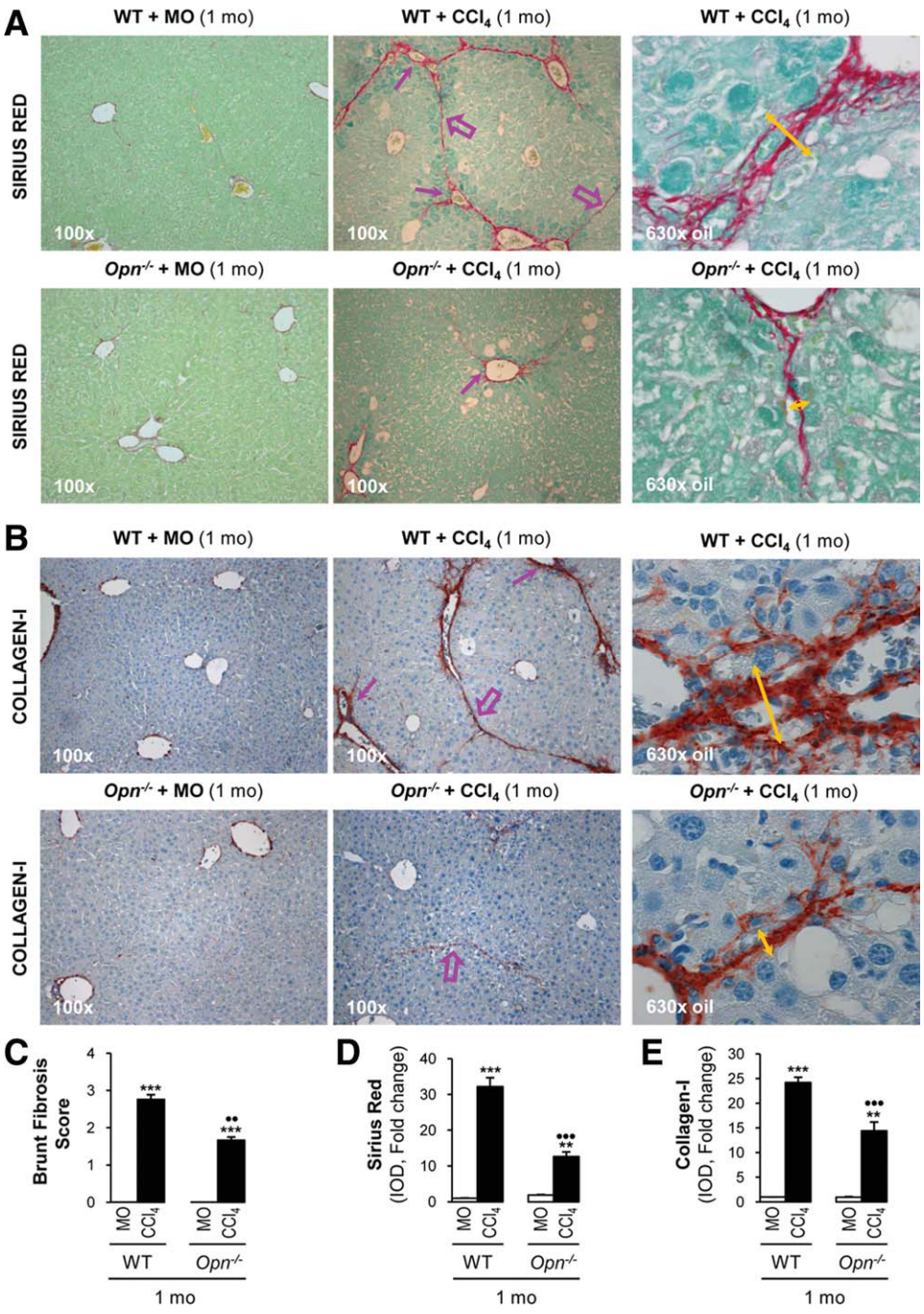

Fig. 7. WT mice show more CCl_4-induced liver fibrosis than $Opn^{-/-}$ mice. C57BL/6J WT and $Opn^{-/-}$ mice were injected CCl_4 or MO for 1 month. Sirius red/fast green staining indicated fibrosis stage ~3 in CCl_4-injected WT and ~1-2 in CCl_4-injected $Opn^{-/-}$ mice (portal ➔ and bridging ⇨ fibrosis) as well as greater scar thickness in WT compared to $Opn^{-/-}$ mice (↔) (A). Collagen-I IHC confirmed the extent of portal fibrosis (➔), bridging fibrosis (⇨) and scar thickness (↔) in CCl_4-injected mice (B). Brunt fibrosis score (C), Sirius red morphometry (D) and Collagen-I morphometry analysis (E). Results are expressed as mean values ± SEM. $n = 8$/group; $^{**}P < 0.01$ and $^{***}P < 0.001$ for CCl_4 versus MO; $^{\bullet\bullet}P < 0.01$ and $^{\bullet\bullet\bullet}P < 0.001$ for $Opn^{-/-}$+CCl_4 versus WT+CCl_4.

treated WT compared to $Opn^{-/-}$, mice, extensive portal fibrosis, bridging fibrosis and a ~3-fold increase in scar thickness (Supporting Fig. 8A, 8B). Thus, fibrosis was more distinct in TAA-treated WT than in $Opn^{-/-}$ mice, as quantified by Brunt fibrosis score and by Sirius red and Collagen-I morphometry (Supporting Fig. 8C-8E).

Collectively, these results suggest that increased OPN expression *per se* or after chronic liver injury and oxidant stress can stimulate Collagen-I deposition *in vivo*. In addition, the *in vitro* studies demonstrate that intracellular OPN plays an autocrine role in regulating Collagen-I expression in HSCs. Moreover, treatment with rOPN to resemble the paracrine actions of secreted OPN increases HSC invasion, chemotaxis and wound-healing potential and up-regulates Collagen-I via integrin $\alpha_v\beta_3$ engagement and activation of PI3K-pAkt-NFκB signaling (Supporting Fig. 9).

Discussion

It is becoming clearer that OPN is significantly induced during liver injury, both in humans and in

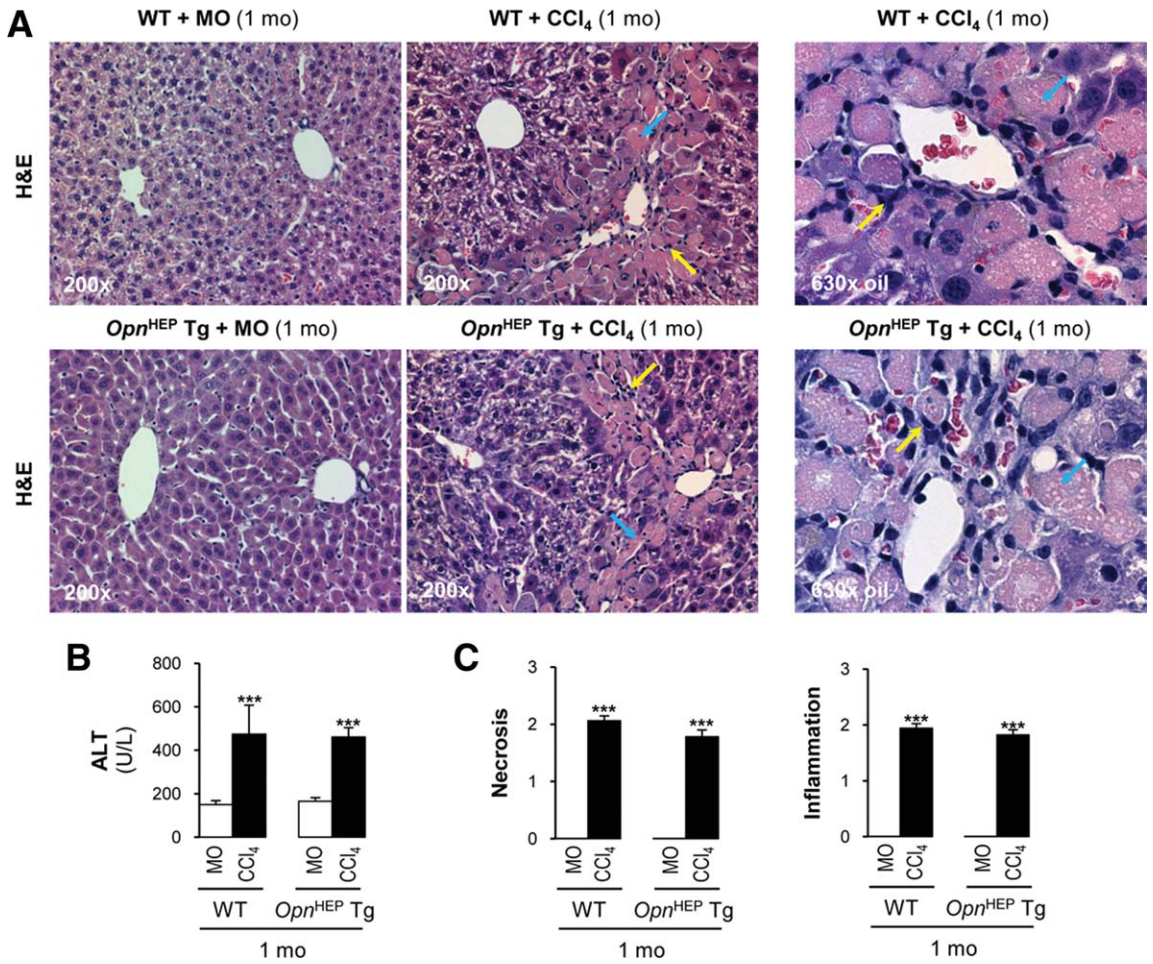

Fig. 8. Opn^{HEP} Tg mice in C57BL/6J genetic background show more CCl_4-induced fibrosis than WT mice. WT and *Opn*^{HEP} Tg mice were injected with MO or CCl_4 for 1 month. H&E staining revealed similar centrilobular necrosis (➔) and inflammation (➔) in CCl_4-injected *Opn*^{HEP} Tg and in WT mice (A). ALT activity (B). Necrosis and inflammation scores (C). Sirius red/fast green staining and IHC for Collagen-I demonstrated more portal fibrosis (➔) bridging fibrosis (⇨) and sinusoidal fibrosis (➔) in CCl_4-injected *Opn*^{HEP} Tg than in WT mice (D and E). Brunt fibrosis score, Collagen-I and Sirius red/fast green morphometry (F). Results are expressed as mean values ± SEM. $n = 8$/group; $^{**}P < 0.01$ and $^{***}P < 0.001$ for CCl_4 versus MO; $^{\bullet\bullet}P < 0.01$ for *Opn*^{HEP} Tg+CCl_4 versus WT+CCl_4.

rodents.[4-6,17] In the past few years, work from several groups[4-6,17] studied the potential role of OPN in liver fibrosis, albeit with inconclusive results. Studies by Lee et al.[5] demonstrated an OPN increase in the culture medium from culture-activated HSCs and under oral CCl_4 administration; however, no mechanistic studies were performed to dissect how OPN regulates Collagen-I protein deposition. Lorena et al.[6] suggested increased susceptibility to CCl_4 injection in $Opn^{-/-}$ mice. The investigators claimed that the protection observed in WT mice was the result of enhancement of hepatocyte survival and reduction in nitric oxide synthase 2 expression; yet, they neither provided IHC for cell-survival markers nor measured the concentration of NO· or $ONOO^-$ to support their conclusions and no studies on Collagen-I regulation were performed. Last, a recent publication from Syn et al.[4] proposes a role for the Hedgehog-signaling pathway in activating OPN and promoting fibrosis progression in nonalcoholic steatohepatitis; however, it is not clear which OPN isoform the investigators were referring to, and it is *Gli1*, and not *Gli2*, expression that is widely considered the most reliable readout for cells undergoing active Hedgehog signaling.

Thus, there is a well-timed need for dissecting the molecular mechanism on how this matricellular protein could regulate the fibrogenic response to liver injury and, specifically, Collagen-I protein expression by HSCs. Currently, there are many unresolved questions on how OPN could induce a feed-forward mechanism to promote scarring and among these are the following: Does extracellular OPN have the ability to increase HSC profibrogenic potential and HSC-derived Collagen-I protein?; which HSC receptors are involved in the profibrogenic cascade triggered by OPN?; what intracellular signals are activated upon OPN-receptor binding that drive the Collagen-I

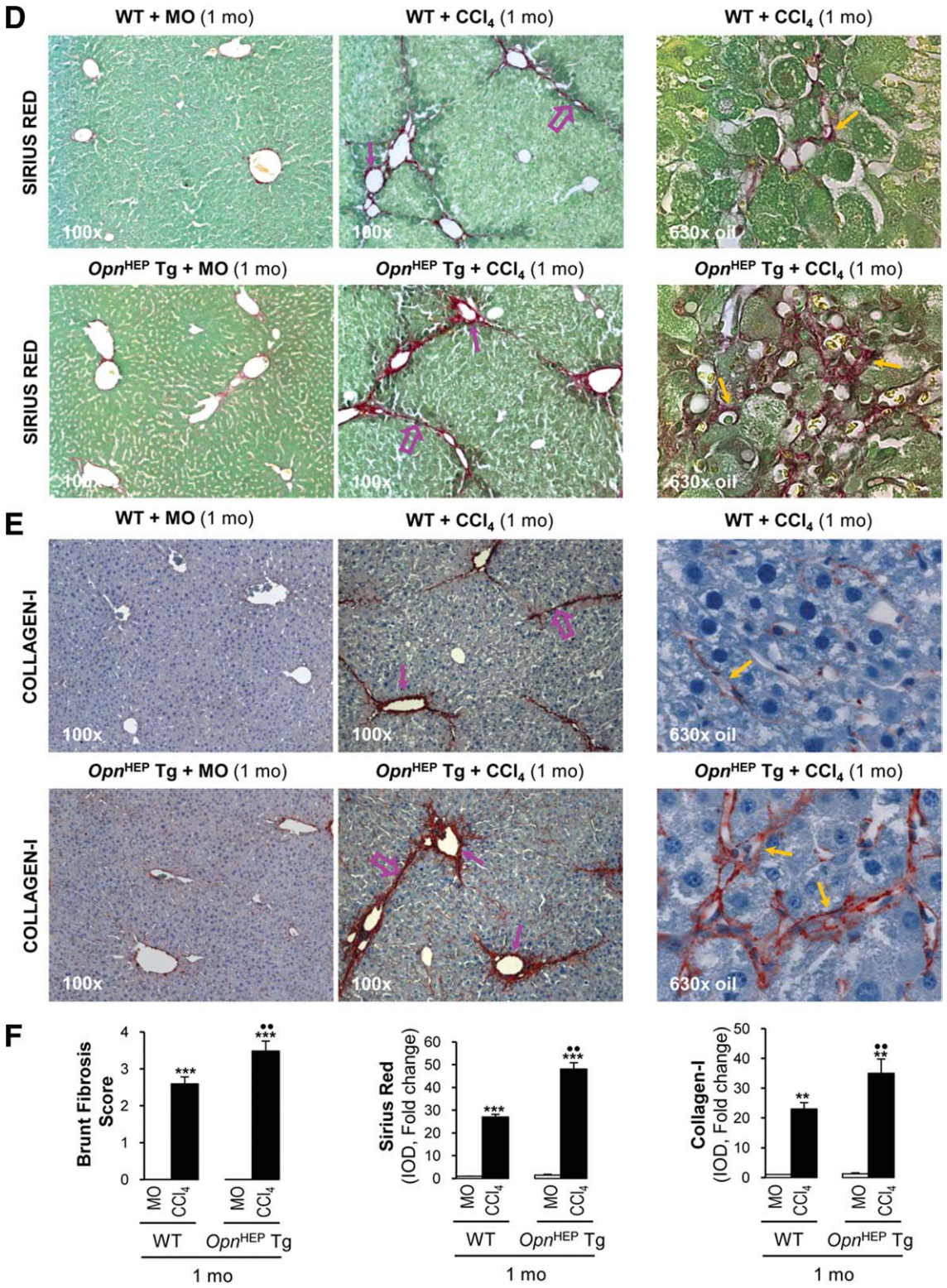

Fig. 8. (Continued).

increase in HSCs?; is autocrine OPN signaling involved in regulating Collagen-I in HSCs?; is OPN sensitive to oxidant stress?; which OPN isoforms appear during the course of liver fibrosis?; and does OPN induce sinusoidal fibrosis?.

Thus, the overall aim of this work was to address these questions, identify the mechanism for the OPN-driven Collagen-I up-regulation in HSCs, and determine the functional role of OPN in the pathogenesis of liver fibrosis. The idea that OPN mediates liver

fibrosis is relevant for several reasons. First, because the observation that OPN is up-regulated in HSCs during hepatic injury provides an excellent conceptual advance in our understanding of liver fibrogenesis, as it appears that OPN up-regulates HSC Collagen-I protein both in an autocrine and paracrine fashion. Second, it supports the clinicopathological finding that injury occurring in the central region is accompanied by fibrosis. Third, it opens the possibility of linking a soluble cytokine/matricellular protein with fibrogenesis. Last, the identification of the mechanism and mediators involved in the profibrogenic actions of OPN could help in devising strategies for therapeutic targeting.

Our *in vitro* experiments validated the hypothesis of the profibrogenic and proinvasive actions of OPN in HSCs. Mechanistic studies identified the HSC membrane proteins engaged by OPN and the proximal signaling molecules/oxidant stress-sensitive kinases activated upon OPN binding that trigger the fibrogenic cascade. The experimental data identified integrin $\alpha_v\beta_3$ as an efficient conveyor of the OPN-mediated profibrogenic actions in HSCs and pointed at PI3K-pAkt activation and the NFκB-signaling pathway as highly involved in this process.

Because OPN signals via integrins and CD44, it is feasible that following liver injury, a ligand for $\alpha_v\beta_3$ integrin, such as OPN, accumulates in the space of Disse and acts in a $\alpha_v\beta_3$ integrin-dependent manner to maintain Collagen-I induction, HSC activation, invasion and migration. Because OPN binds ECM proteins,[35,36] this binding ability may enhance HSC activation, migration and invasion—key HSC features for the development of fibrosis. The finding that blocking CD44 did not prevent the effect of rOPN on Collagen-I may be related to the ability of hyaluronic acid—a glycosaminglycan synthesized during HSC activation[24,37]—to bind CD44; thus, competitive inhibition between hyaluronic acid and rOPN for CD44 binding could occur in HSCs, although this possibility needs further investigation.

Several observations support the role for PI3K-pAkt activation and the NFκB-signaling pathway in the effects mediated by rOPN on Collagen-I. First, rOPN rapidly increased PI3K, the ratios pAkt ^{473}Ser/Akt, pIKKα,β $^{176/180}$Ser/IKKα,β and pIκBα ^{32}Ser/IκBα as well as nuclear translocation of p65. Second, inhibitors of PI3K activation and NFκB signaling blunted the rOPN-mediated increase in intra- and extracellular Collagen-I protein. Third, blockade of $\alpha_v\beta_3$ integrin signaling with a neutralizing Ab and incubation with wortmannin or LY294002 prevented the induction of PI3K, the increase in the ratios pAkt ^{473}Ser/Akt, pIKKα,β $^{176/180}$Ser/IKKα,β and pIκBα ^{32}Ser/IκBα, nuclear translocation of p65 and the up-regulation of Collagen-I protein by rOPN. Involvement of the mTOR cascade was ruled out, because rOPN altered neither mTOR-p706SK expression nor mTOR phosphorylation. Therefore, this study linked extracellular and/or secreted OPN (i.e., paracrine effect) with $\alpha_v\beta_3$ integrin binding, PI3K-pAkt activation, NFκB signaling and scarring.

Work from several laboratories,[3-6] including our own, suggests that HSCs are an important source of OPN during liver injury. To date, OPN was believed to exert its effects by binding the RGD motif in integrins and the cell-surface receptor CD44; however, an intracellular function of OPN in liver fibrosis was largely unknown. Because HSCs isolated from *Opn*$^{-/-}$ mice were less profibrogenic than those from WT mice and infection of HSCs with Ad-OPN increased intracellular Collagen-I, these results suggested a novel autocrine mechanism whereby intracellular OPN could modulate Collagen-I deposition in HSCs. Alternatively, extracellular OPN, either from HSCs or from neighboring cells, may activate HSCs through its receptor ($\alpha_v\beta_3$ integrin), as suggested above, thus creating a positive feedback loop.

To further validate our hypothesis, we then assessed whether OPN contributed to the fibrogenic response *in vivo* using two mouse models of drug-induced liver injury. The data from human samples and from the mouse models showed that most of the OPN found in liver injury appeared to have been cleaved at least at the endpoint of the experiments. The role of each cleaved isoform in regulating the fibrogenic response to liver injury, as well as the identification of the proteases that cleave hepatic OPN, is currently under active investigation in our laboratory, because additional integrin-binding sites, other than $\alpha_v\beta_3$ integrin, are likely to be uncovered by proteolytic processing of the protein.

Upon the onset of liver injury in mice, the increase in OPN likely results from oxidant stress because CCl_4 and TAA metabolism via cytochrome P450s generate a considerable amount of free radicals[33] and the *in vitro* data demonstrated the OPN responsiveness to oxidant stress, which was blocked by antioxidant treatment. Furthermore, cotreatment with SAM, known to elevate GSH levels, prevented the increase in OPN and the fibrogenic response in WT mice injected with CCl_4 for 1 month.

Although it is possible that the chronic effects of *Opn* ablation could be secondary to its effects on liver injury itself (i.e., inflammation and ductular reaction, unpublished observations), the data clearly reveal a direct action of OPN on Collagen-I protein

expression, a key event in liver fibrosis. Hence, OPN appears to induce scarring *per se*. This is, indeed, also supported by the finding that though ALT activity and the necrosis and inflammation scores were similar, there was increased portal, bridging and sinusoidal fibrosis, along with enhanced width of the collagenous septa in CCl_4-injected Opn^{HEP} Tg mice, compared to their WT littermates. Notably, Opn^{HEP} Tg mice developed spontaneous fibrosis over time, whereas WT mice did not. Last, in line with the results using Opn^{HEP} Tg mice and the *in vitro* data, fibrilar Collagen-I content and scar thickness was significantly lowered by OPN ablation *in vivo*. It is likely that secreted OPN allows paracrine signaling to HSCs, whereas endogenous OPN expression in HSCs signals in an autocrine fashion, amplifying fibrogenic response. The cell- and matrix-binding ability of OPN may also facilitate a proper stromal and fibrillar collagen network organization. Overall, it is reasonable to propose that OPN may drive the fibrogenic response, among others, by directly regulating Collagen-I deposition. Thus, OPN emerges as a key soluble cytokine and ECM-bound molecule promoting liver fibrosis.

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