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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Periostin promotes hepatic fibrosis in mice by modulating hepatic stellate cell activation via α_v integrin interaction

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Abstract

Background Periostin is a matricellular protein that serves as a ligand for integrins and is required for tissue remodeling and fibrosis. We investigated the role of periostin in hepatic fibrosis and the mechanisms involved.

Methods Primary hepatic stellate cells (HSCs) and the HSC-immortalized cell line LX2 were used to study the profibrotic property of periostin and the interaction of periostin with integrins. Wild-type and periostin-deficient (periostin$^{-/-}$) mice were subjected to two distinct models of liver fibrosis induced by hepatotoxic (carbon tetrachloride or thioacetamide) or cholestatic (3.5-diethoxycarbonyl-1.4-dihydrocollidine) injury.

Results Periostin expression in HSCs and LX2 cells increased in association with their activation. Gene silencing of periostin resulted in a significant reduction in the levels of profibrotic markers. In addition to enhanced cell migration in response to periostin, LX2 cells incubated on periostin showed significant induction of α-smooth muscle actin and collagen, indicating a profibrotic property. An antibody targeting $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins suppressed cell attachment to periostin by 60 and 30 % respectively, whereas anti-$\alpha_5\beta_1$ antibody had no effect. Consistently, α_v integrin-silenced LX2 cells exhibited decreased attachment to periostin, with a significant reduction in the levels of profibrotic markers. Moreover, these profibrotic effects of periostin were observed in the mouse models. In contrast to extensive collagen deposition in wild-type mice, periostin$^{-/-}$ mice developed less noticeable hepatic fibrosis induced by hepatotoxic and cholestatic liver injury. Accordingly, the profibrotic markers were significantly reduced in periostin$^{-/-}$ mice.

Conclusion Periostin exerts potent profibrotic activity mediated by α_v integrin, suggesting the periostin–α_v integrin axis as a novel therapeutic target for hepatic fibrosis.

Keywords Matricellular protein · Liver fibrogenesis · Cell adhesion receptor · Myofibroblasts · Cell migration

Abbreviations

Col1α1	Collagen type I, alpha 1
DDC	3,5-Diethoxycarbonyl-1,4-dihydrocollidine
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
FBS	Fetal bovine serum
HSC	Hepatic stellate cell
siRNA	Small interfering RNA
α-SMA	α-Smooth muscle actin

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TAA Thioacetamide
TGF Transforming growth factor
TIMP-1 Tissue inhibitor of metalloproteinase 1

Introduction

Periostin (also known as osteoblast-specific factor 2) is a 90-kDa secreted matricellular protein originally isolated from a mouse osteoblast cell line [1, 2]. Like other matricellular proteins, periostin interacts with cell surface receptors and the structural components of the extracellular matrix (ECM). Periostin is assigned to the fasciclin family; it contains four tandem fasciclin domains that modulate signal transduction for cell proliferation, migration, and differentiation by interacting with various integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_4$, and $\alpha_M\beta_2$) [3]. At its N-terminus, periostin has an EMI domain consisting of a small cysteine-rich module of approximately 75 amino acids that binds to collagen type I and fibronectin, contributing to the organization of ECM architecture.

Recently, increasing evidence regarding the role of periostin in tissue wound repair and fibrogenesis has been reported [4]. In the heart, periostin is strongly induced by myocardial infarction or long-term pressure overload, and it promotes myocardial repair [5–8]. In patients with idiopathic pulmonary fibrosis, periostin expression is increased within the regions of active fibrosis, and pulmonary fibroblasts have been identified as a major source of this protein [9]. Analysis of periostin-deficient (periostin$^{-/-}$) mice has revealed that periostin is required for both dermal wound repair and bleomycin-induced pulmonary fibrosis [10, 11]. Periostin is also involved in the pathogenesis of proliferative vitreoretinopathy, in which excessive scar tissue grows over the surface of the retina and into the vitreous humor [12]. Taken together, these reports suggest that periostin promotes wound healing by modulating the actions of tissue-resident fibroblasts.

Liver cirrhosis represents the end stage of hepatic fibrosis, and it is an increasing reason for morbidity and mortality worldwide [13]. In an attempt to develop effective antifibrosis therapy, the origin of myofibroblasts (the main source of ECM) has been extensively studied, with the main candidates being activated hepatic stellate cells (HSCs) or activated portal fibroblasts, depending on the cause of liver injury [14]. Activated HSCs are widely recognized as the main cells contributing to liver fibrosis associated with hepatotoxicity. Although activated portal fibroblasts are implicated in the early stages of cholestatic fibrosis induced by bile duct ligation, the contribution of portal fibroblasts decreases with the progression of liver damage, and HSCs predominate, suggesting that HSCs are the chief contributors to hepatic fibrosis, independent of its cause [14, 15]. It has recently been demonstrated that HSCs express integrins, which play an important role in regulating their various functions, including migration, proliferation, and survival [16, 17]. In addition, an $\alpha_v\beta_3$ integrin antagonist significantly inhibited the synthesis of procollagen type I by HSCs [18]. Since periostin interacts with integrins, these observations suggest that periostin may regulate the development of hepatic fibrosis by modulating HSC function. Indeed, recent studies have demonstrated that periostin knockdown in HSCs attenuates the profibrotic properties in response to transforming growth factor (TGF)-β_1 in vitro [19]. In addition, periostin$^{-/-}$ mice exhibited resistance to experimental liver fibrosis induced by either CCl_4 or a diet deficient in methionine and choline [20, 21].

In the present study we investigated whether periostin modulated the biological functions of HSCs in vitro, with a particular focus on its interaction with integrins, which are known receptors for periostin. We also performed in vivo experiments using periostin$^{-/-}$ mice to further verify the biological significance of periostin in the development of liver fibrosis induced by both hepatotoxic inhury and cholestatic injury.

Materials and methods

Mouse models of liver fibrosis

Male periostin$^{-/-}$ mice (C57BL/6 background, 8–10 weeks old) were prepared as previously described [22], and conventional C57BL/6 mice were obtained from Hiroshima Jikken Doubutu (Hiroshima, Japan). Acute liver injury was induced by a single intraperitoneal injection of 25 % solution of CCl_4 (Wako, Osaka, Japan) in sterile olive oil (0.5 mL CCl_4/kg body weight), and samples were harvested at 1, 3, 7, or 14 days after the treatment. To identify the role of periostin in hepatic fibrosis, mice were subjected to hepatotoxic liver injury (CCl_4 or thioacetamide, TAA; Wako, Osaka, Japan) or cholestatic liver injury (3,5-diethoxycarbonyl-1,4-dihydrocollidine, DDC; Sigma-Aldrich, St Louis, MO, USA). In the chronic hepatotoxicity models, mice were treated by intraperitoneal injection of CCl_4 (0.5 mL CCl_4/kg body weight) twice weekly for 4 weeks or were given TAA dissolved in drinking water at a concentration of 0.3 g/L for 16 weeks. In the cholestatic injury model, mice were fed a 0.1 % DDC-containing diet for 4 weeks. All animal protocols were approved by the Institutional Animal Care and Use Committee of Hiroshima University.

Isolation of HSCs and cell culture

Rat primary HSCs were isolated from 20-week-old male Sprague Dawley rats (Hiroshima Jikken Doubutsu, Hiroshima, Japan) as previously described [23]. Primary HSCs and LX2 cells, immortalized human activated HSCs (a gift from Scott L. Friedman, Mount Sinai Hospital, New York, NY, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10 % fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA) and 1 % penicillin–streptomycin (Life Technologies, Carlsbad, CA, USA) [24].

Gene silencing by small interfering RNA

Small interfering RNAs (siRNAs) specific to human periostin, α_v integrin (Invitrogen, Grand Island, NY, USA), and a nonsilencing negative control (Sigma-Aldrich Japan, Hokkaido, Japan) were transfected with use of Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA). The siRNA sequences used in this study were as follows: human periostin, 5′-rGrArCrArArCrArArAUrGrGUrGUrArAUUTT-3′ and 5′-rArAUUrArCrArCrCrAUUUrGUUrGUrCTT-3′; human α_v integrin, 5′-GGUCCAAGUUCAUUCAGCAAGGCAA-3′ and 5′-UUGCCUUGCUGAAUGAACUUGGACC-3′. To investigate whether silencing of periostin induces HSC apoptosis, an immunoassay for single-stranded DNA in HSCs following siRNA transfection was performed with an ApoStrand™ ELISA apoptosis detection kit (Enzo Life Sciences. Farmingdale, NY, USA).

Flow cytometry

Flow-cytometric analysis for the expression of $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$ integrins was performed with primary antibodies against integrin $\alpha_v\beta_3$ (LM609; Merck Millipore, Tullagreen, Ireland), α_5 (JBS5; Abcam, Cambridge, UK), and $\alpha_v\beta_5$ (ALULA; a gift from Dean Sheppard, University of California, San Francisco, USA), with goat anti-mouse phycoerythrin (Santa Cruz Biotechnology, Dallas, TX, USA) as a secondary antibody. Expression profiles were acquired with a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

Scratch assay

Confluent cultures of LX2 cells incubated for 24 h in a serum-free medium were scratched with a sterile pipette tip, and were incubated with either phosphate-buffered saline or a 5 μg/mL solution of recombinant human periostin (R&D Systems, Minneapolis, MN, USA) and photographed under a phase contrast microscope at 24 h.

Transwell migration assay

The undersides of the polycarbonate membranes with 8-μm pores of a Transwell insert (Corning, Corning, NY, USA) were coated with a 5 μg/mL solution of recombinant human periostin or phosphate-buffered saline. A total of 4×10^4 LX2 cells were incubated with serum-free DMEM for 24 h and added to the top of each chamber. The cells were then allowed to migrate to the lower chambers containing 1 % FBS–DMEM for 24 h. Cells that had migrated on the underside of the membrane were fixed in 1 % formalin and stained with crystal violet for cell counting.

Cell adhesion assay

A cell adhesion assay was performed as described in [25], with slight modifications. Briefly, 96 wells of a MaxiSorp NUNC-Immuno plate (Thermo Scientific, Waltham, MA, USA) were coated with a 5 μg/mL solution of recombinant human periostin or a 10 μg/mL solution of poly(L-lysine) (Sigma-Aldrich, St Louis, MO, USA) overnight at 4 °C. Following 1 h of incubation at 37 °C with 1 % bovine serum albumin (Nacalai Tesque, Kyoto, Japan) in DMEM, the plates were filled with 50 μL of LX2 cell suspension (10×10^5 cells per milliliter in DMEM) with or without neutralizing antibodies, and centrifuged at 1000*g* for 5 min. The plates were then incubated for 1 h at 37 °C. Unattached cells were removed by centrifugation upside down at 1000*g* for 5 min, followed by cell counting.

Hydroxyproline assay

Liver tissues (30 mg/300 μL in sterilized water) were hydrolyzed with 6 N HCl for 3 h at 120 °C. The precipitates were removed by a MILLEX-HV 0.45-μm filter unit (Merck Millipore, Darmstadt, Germany), and the hydroxyproline concentration was quantified with a hydroxyproline colorimetric assay kit (BioVision, Milpitas, CA, USA).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed as described previously with β-actin as an internal control [26]. The sequences of specific primers are listed in Table S1.

Western blot analysis

Cells and liver tissues were lysed with radioimmunoprecipitation assay buffer [0.1 % sodium dodecyl sulfate, 0.5 % deoxycholic acid, 1 % NP-40, 150 mM NaCl, and 50 mM tris(hydroxymethyl)aminomethane–Cl, pH 7.6] and centrifuged at 10,000*g* for 10 min. The supernatant

was subjected to Western blot analysis with the primary antibodies against α-smooth muscle actin (α-SMA; ab15734, Abcam, Cambridge, UK; A5228, Sigma-Aldrich, St Louis, MO, USA), periostin (ab14041 and ab92460; Abcam), collagen type I (ab 59435 and ab34710; Abcam), and glyceraldehyde-3 phosphate dehydrogenase (G9545; Sigma-Aldrich, St Louis, MO, USA).

Histological and immunohistochemistry examination

Liver specimens were fixed in 4 % paraformaldehyde and embedded in paraffin. Then 4-μm thick sections were subjected to hematoxylin and eosin staining or azan staining. Immunohistochemistry was performed with the antibody against periostin (ab14041; Abcam) or α-SMA.

Analytical techniques

Plasma periostin levels were measured by a sandwich ELISA with originally developed anti-periostin monoclonal antibody (clone no. SS19D) [27]. The concentrations of periostin in the conditioned medium were measured with a human periostin ELISA kit (Shino-Test, Tokyo, Japan). Plasma concentrations of aspartate aminotransferase and alanine aminotransferase were determined enzymatically.

Statistical analysis

Data were analyzed by two-way ANOVA or two-sided, unpaired Student's *t* tests. The results are expressed as the mean ± standard error of the mean. We considered values to be significant when $P < 0.05$.

Results

Priostin is upregulated in fibrotic liver in vivo and induction of periostin is associated with HSC activation in vitro

To investigate the contribution of periostin to liver fibrosis, we induced liver fibrosis in mice using CCl_4 or bile duct ligation and studied the expression of periostin in the fibrotic livers. In the homogenates of the livers from the two models, both the gene expression and the protein expression of periostin were significantly elevated (Fig. S1). The serum level of periostin was also significantly higher in CCl_4-treated mice than in untreated mice (107.51 ± 61.47 ng/mL vs 26.78 ± 25.20 ng/mL; $P < 0.01$). Next, we analyzed the time course of the increase in periostin expression following a single administration of CCl_4 at days 0, 1, 3, 7, and 14. Centrilobular necrosis was most prominent at day 1, along with significant increases in the serum levels of aspartate aminotransferase and alanine aminotransferase (Fig. S2a, b). Periostin gene expression peaked at day 3, in the same manner as collagen type I, alpha 1 (Col1α1) gene expression. In contrast, the gene expression of α-SMA, a marker of HSC activation, peaked as early as day 1 after administration along with the gene expression of the inflammatory cytokines interleukin-6 and tumor necrosis factor α. The protein level of periostin also became most elevated after that of α-SMA became most abundant (Fig. S2c, d). These results suggest that similarly to collagen type I, periostin induction in the liver requires the activation of HSCs.

Since periostin is generally secreted by tissue fibroblasts or the cells that undergo fibroblast-like transformation in response to TGF-β_1 [28], we speculated that main source of periostin in the liver might be activated HSCs. To confirm this, LX2 cells, a human HSC cell line, were treated with recombinant TGF-β_1. Following TGF-β_1 stimulation, periostin gene expression gradually increased up to about fivefold for 48 h (Fig. 1a). This increase was confirmed at the protein level in cells and in the culture medium for secreted periostin (Fig. 1b, c). These changes were parallel to the increase in α-SMA level. With the plate activation of rat primary HSCs, an increase in periostin expression was also observed, as with α-SMA and Col1α1 (Fig. 1d, e). These in vitro results demonstrated that HSC activation regardless of direct stimulation of TGF-β induced the expression of periostin.

Silencing endogenous periostin in HSCs reduces profibrotic phenotype

To investigate the effect of downregulation of periostin in LX2 cells that recapitulates many features of an activated HSC phenotype, we performed knockdown using siRNA. Periostin messenger RNA was effectively downregulated after 24 h, and the minimum periostin protein level was seen after 120 h (Fig. 2a). At this time, the gene expression of profibrotic markers, including α-SMA, Col1α1, and tissue inhibitor of metalloproteinase 1 (TIMP-1), was also significantly downregulated (Fig. 2b). Periostin knockdown did not induce HSC apoptosis, suggesting the possibility that targeted deletion of endogenous periostin deactivated HSCs by interfering with their autocrine loop (Fig. 2c). As previous studies have indicated that periostin acts downstream of TGF-β [2], we investigated the influence of TGF-β_1 on LX2 cells, with or without periostin disruption. Although TGF-β_1 treatment increased α-SMA gene expression in LX2 cells with normal periostin expression, there was no change in LX2 cells with the disruption of periostin (Fig. 2d). This result suggests that periostin is required for the activation of HSCs by TGF-β_1.

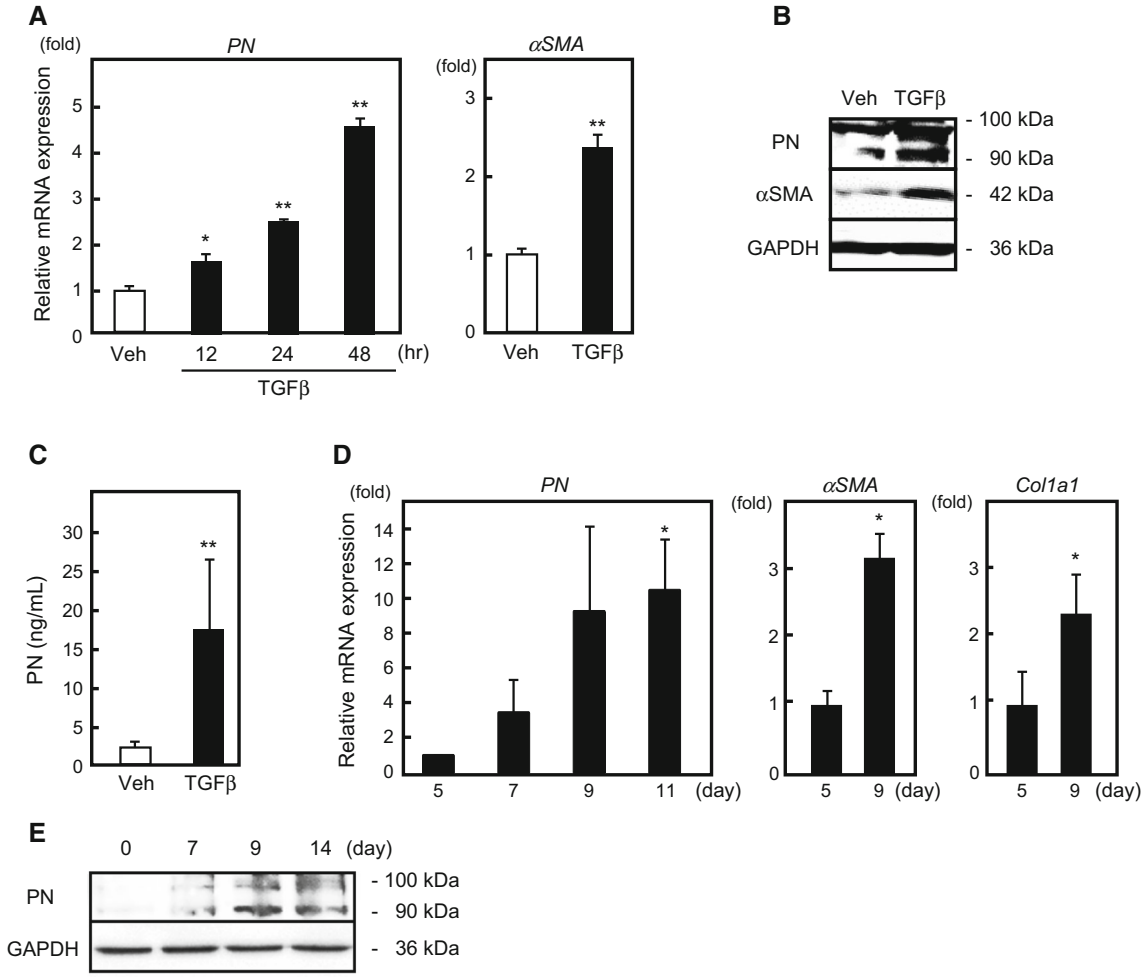


Fig. 1 Periostin induction is associated with activation of hepatic stellate cells (HSCs) in vitro. **a** A human HSC line, LX2, was stimulated with a 10 ng/mL solution of transforming growth factor β (*TGFβ*) or vehicle (*Veh*), and periostin (PN) transcripts were quantified by quantitative real-time polymerase chain reaction ($n = 4$) at the indicated time points. **b** Protein expression of PN was analyzed by Western blot analysis (TGFβ, 10 ng/mL, 72 h). **c** PN concentrations of cultured media in which LX2 cells were stimulated by TGFβ (10 ng/mL, 72 h) were measured by ELISA. **d**, **e** Rat primary HSCs were activated by culture on a plastic dish and PN expression was analyzed by quantitative real-time polymerase chain reaction ($n = 4$) and immunoblotting. Data are expressed as the mean ± standard error of the mean. *$P < 0.05$ versus Veh on day 5, **$P < 0.01$ versus Veh on day 5, *αSMA* α-smooth muscle actin, *Col1a1* collagen type I, alpha 1, *GAPDH* glyceraldehyde-3 phosphate dehydrogenase, *mRNA* messenger RNA

Periostin enhances HSC motility and activation

To evaluate the effect of periostin on HSC motility, we assessed cell migration by the scratch wound closure assay and the Transwell migration assay. Figure 3a shows the enhanced nondirectional migration of HSCs into the scratch area after stimulation with recombinant periostin. In the Transwell migration assay, our coating the underside of the chamber membrane with recombinant periostin led to a twofold increase in directional cell migration (Fig. 3b). These findings strongly suggest that periostin promotes HSC migration. To assess the influence of periostin on HSC activation, we first studied whether HSCs interacted with periostin by performing a cell adhesion assay. As shown in Fig. 3c, the binding of LX2 cells to periostin increased in a dose-dependent manner, suggesting that HSCs express cell adhesion molecules for periostin. LX2 cells cultured on periostin-coated plates showed a marked increase in the protein expression of profibrotic markers (α-SMA and collagen type I) and endogenous periostin as compared with poly(L-lysine), which is known to enhance cell adhesion via electrostatic bound formation, Together with the observation in Fig. 2, these results suggest that periostin promotes HSC activation via a positive feedback loop (Fig. 3d).

Periostin interacts with α_v integrin

As periostin is a ligand for integrins, which are reported to play a role in collagen synthesis by HSCs [17], we

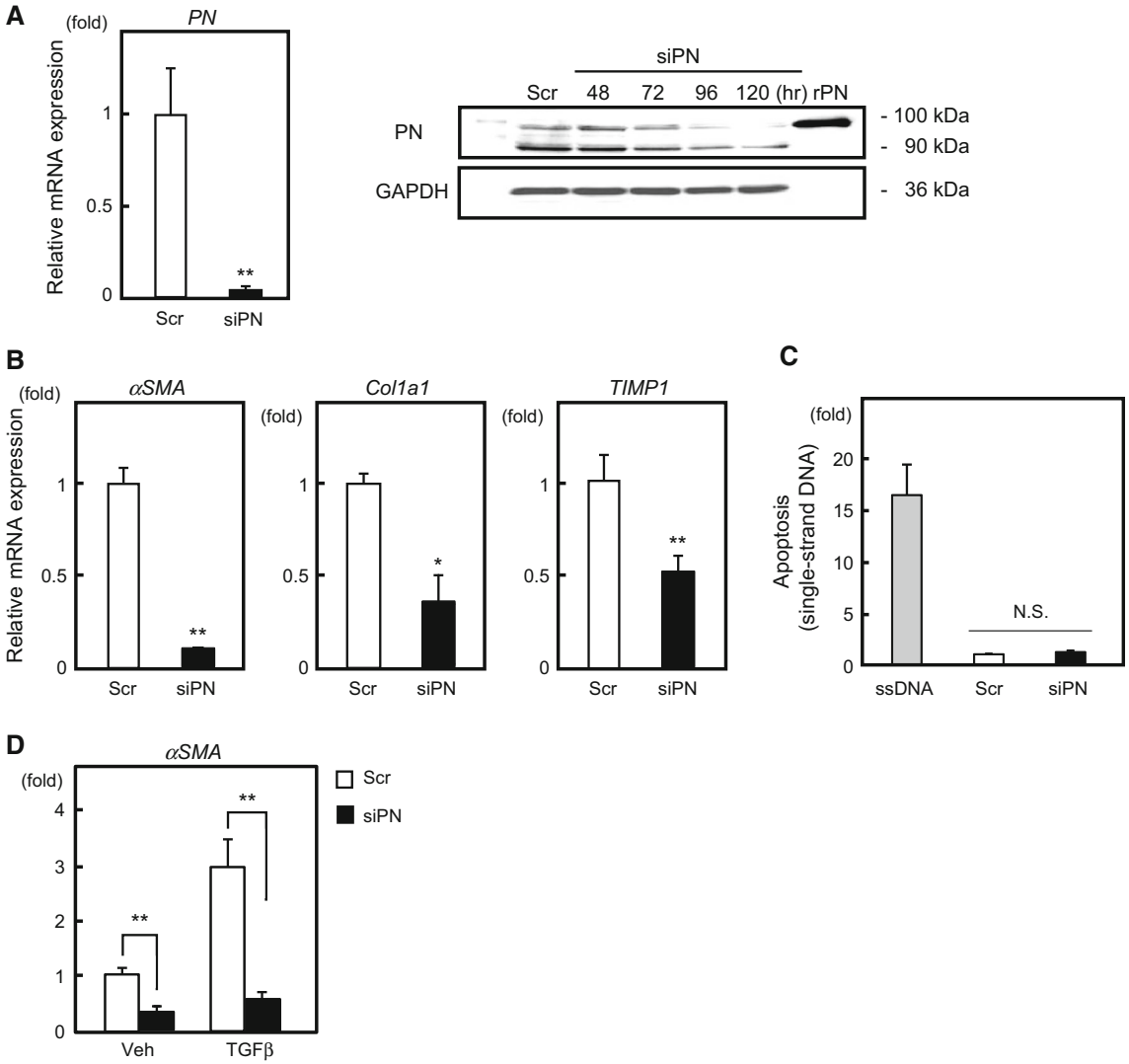


Fig. 2 Periostin knockdown in hepatic stellate cells reduces the levels of fibrosis markers. LX2 cells were transfected with either small interfering RNA (siRNA) targeting human periostin (*siPN*) or nontargeting scramble control (*Scr*). **a** Knockdown of periostin (*PN*) was confirmed by quantitative real-time polymerase chain reaction ($n = 5$) after 24 h of siRNA transfection and by Western blot analysis at a different time point. **b** Gene expression of fibrosis markers was analyzed by quantitative real-time polymerase chain reaction 120 h after siRNA transfection. **c** Single-stranded DNA (*ssDNA*) in LX2 cells after 120 h of siRNA transfection was quantified with an ELISA kit ($n = 8$). **d** LX2 cells transfected with either siPN (*black bar*) or nontargeting control RNA (*white bar*) were treated with transforming growth factor β (*TGFβ*; 10 ng/mL, 48 h), and the messenger RMA (*mRNA*) level of α-smooth muscle actin (*αSMA*) was quantified by quantitative real-time polymerase chain reaction ($n = 6$). Data are expressed as the mean ± standard error of the mean. *One asterisk* $P < 0.05$, *two asterisks* $P < 0.01$, *Col1a1* collagen type I, alpha 1, *GAPDH* glyceraldehyde-3 phosphate dehydrogenase, *N.S.* not significant, *rPH* recombinant periostin, *TIMP1* tissue inhibitor of metalloproteinase 1, *Veh* vehicle

hypothesized that periostin-induced activation of HSCs is mediated by integrins. We performed a flow-cytometric analysis of LX2 cells to confirm the expression of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins, which are known receptors for periostin (Fig. 4a). To further assess the role of each integrin, cell adhesion assays were performed with or without neutralizing antibodies against $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins. As shown in Fig. 4b, an antibody against $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins significantly suppressed LX2 cell attachment to periostin by approximately 60 and 30 % respectively, and an additive effect was observed when these neutralizing antibodies were simultaneously added. In contrast, anti-$\alpha_5\beta_1$ antibody had no effect. Consistent with these results, LX2 cells incubated with neutralizing antibodies against $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin had a relatively compact spherical morphology, which contrasted with their flattened and spreading shape after incubation with the anti-$\alpha_5\beta_1$ antibody or control IgG (Fig. 4c). The release of

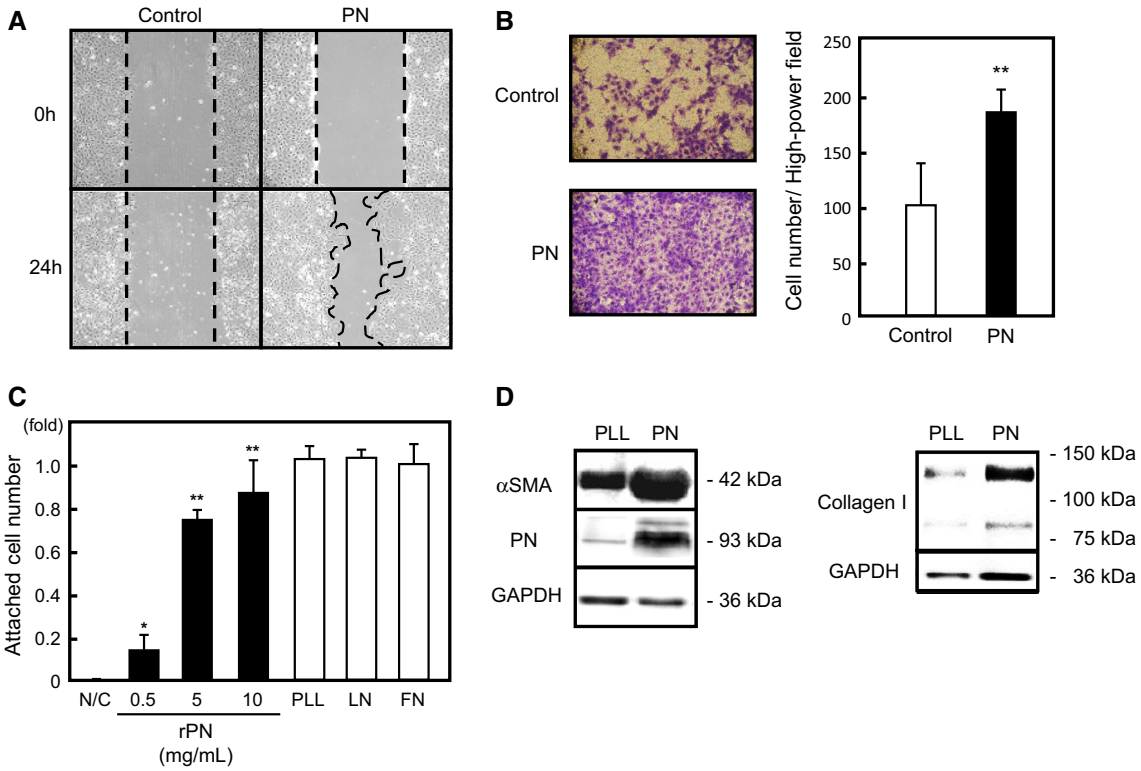


Fig. 3 Periostin (*PN*) enhances hepatic stellate cell motility and activation. **a** LX2 cells were plated at equal density and allowed to adhere to the culture dish for 24 h. A linear scratch was applied to the monolayer with a 200-μL pipette chip. Cells were cultured with recombinant PN (*rPN*; 5 μg/mL, 24 h), and cell motility was assessed by phase contrast microscopy. **b** LX2 cells were plated in a serum-free medium on top of polycarbonate membranes with 8-μm pores of a Transwell insert, the undersides of which were coated with rPN (5 μg/mL) or phosphate-buffered saline. The LX2 cells were allowed to migrate to the lower chambers containing 1 % fetal bovine serum–Dulbecco's modified Eagle's medium for 24 h, followed by cell counting on the underside of the membrane at six randomly selected areas. **c** LX2 cells were plated on rPN (0.5–10 μg/mL), poly(L-lysine) (*PLL*), or bovine serum albumin (negative control, *N/C*)-coated plates and incubated for 1 h. After removal of unattached cells by centrifugation (upside down), the number of attached cell was determined by absorbance ($n = 6$). **d** LX2 cells were plated on rPN (5 μg/mL) or a PLL-coated plate and incubated for 48 h. Immunoblotting was performed for the detection of α-smooth muscle actin (*αSMA*), collagen type I, and endogenous PN. Data are expressed as the mean ± standard error of the mean. *One asterisk* $P < 0.05$ versus control, *two asterisks* $P < 0.01$ versus control, *GAPDH* glyceraldehyde-3 phosphate dehydrogenase

collagen into the culture medium was also reduced by anti-integrin antibodies (data not shown). These results suggest a critical role for α_v integrin in the activation of HSCs.

Periostin activates HSCs by interacting with α_v integrin

To further assess the interaction of periostin with the α_v integrin, LX2 cells were transduced with siRNAs targeting α_v integrin, and successful knockdown was achieved (Fig. 5a). As shown in Fig. 5b, the knockdown of α_v integrin dramatically inhibited the attachment of LX2 cells to periostin by approximately 90 %, as assessed by a cell adhesion assay. The morphological changes of LX2 cells with α_v knockdown on periostin-coated plates (Fig. 5c) were similar to those observed following incubation with neutralizing antibodies against α_v integrins (Fig. 4c). Furthermore, the levels of profibrotic markers, α-SMA, Col1α1, and TIMP-1, were significantly reduced by the disruption of α_v integrin, suggesting that the interaction between periostin and α_v integrin is critical for the activation of HSCs.

Deletion of periostin in mice reduces susceptibility to hepatotoxic liver fibrosis

On the basis of our in vitro observation that periostin enhances the motility and activity of HSCs, we hypothesized that the deletion of periostin in mice would confer resistance to experimental hepatic fibrosis. Figure 6a displays representative histological findings in the livers of wild-type and periostin$^{-/-}$ mice after the administration of either CCl_4 or TAA. In both models of chronic hepatotoxicity, wild-type mice exhibited extensive collagen deposition in the liver, with an increase in the levels of activated HSCs expressing α-SMA mainly in the fibrotic

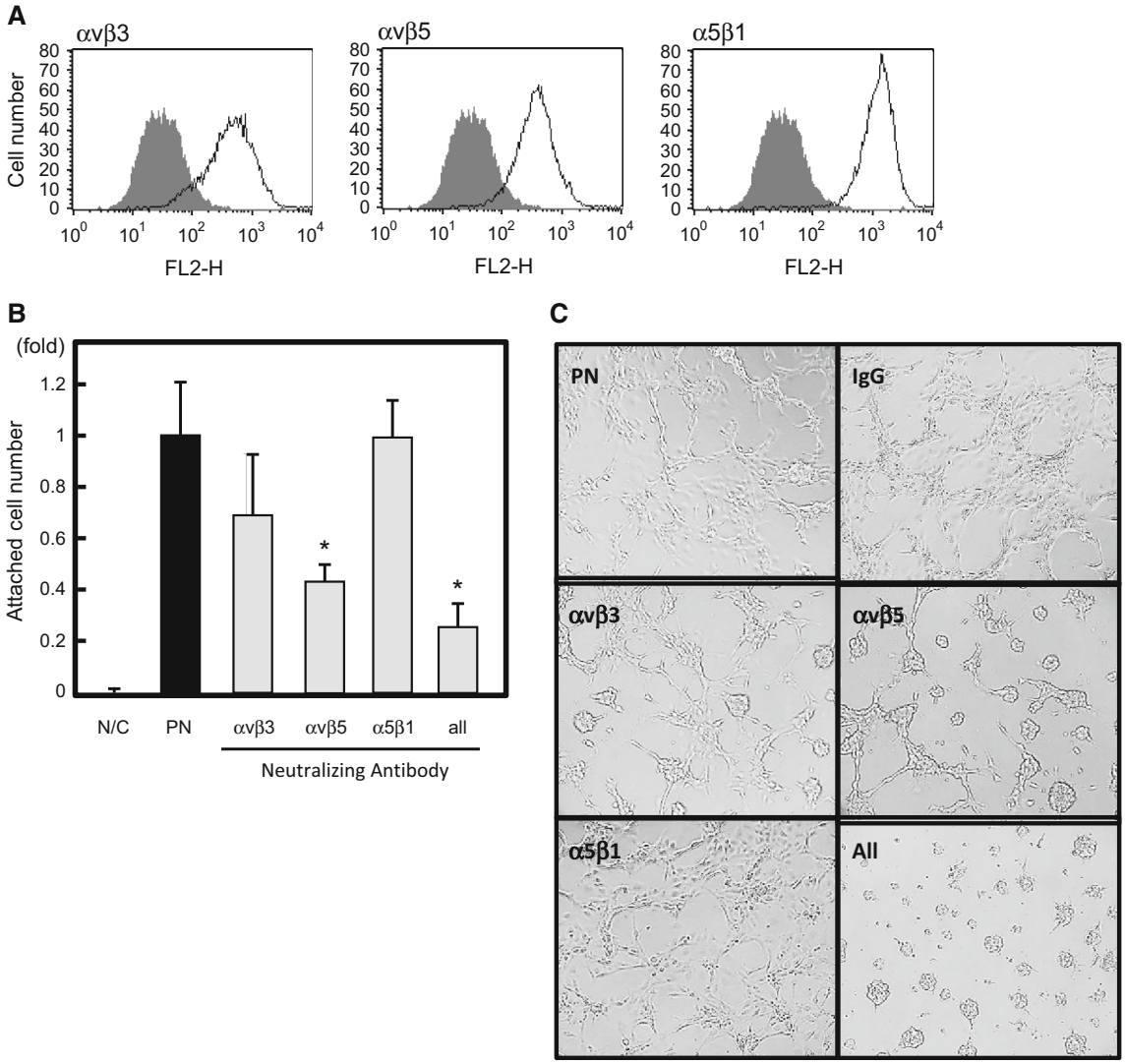


Fig. 4 Periostin (*PN*) interacts with α_v integrin. **a** Surface expression of $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$ integrins on LX2 was analyzed by flow cytometry. **b** LX2 cells were plated on plates coated with a 5 μg/mL solution of recombinant PN in the presence or absence of a neutralizing antibody against $\alpha_v\beta_3$, $\alpha_5\beta_1$ or $\alpha_v\beta_5$ integrin, or in a combination, and incubated for 2 h. After removal of unattached cells by centrifugation (upside down), the number of attached cell was determined by absorbance. **c** Morphological characteristics of LX2 cells cultured on PN-coated plates with or without neutralizing antibodies against integrins (original magnification ×100). Data are expressed as the mean ± standard error of the mean. *Asterisk* $P < 0.05$ versus PN, *N/C* negative control

septa, whereas the changes were much less apparent in periostin$^{-/-}$ mice. It was unexpected that there was no staining for periostin on immunohistochemistry in either the CCl_4 model or the TAA model (data not shown). There was a significant reduction of the hydroxyproline content in periostin$^{-/-}$ mice (Fig. 6b), along with significantly reduced expression of collagen type I and α-SMA (Fig. 6c). Quantitative polymerase chain reaction analysis confirmed reduced transcripts of profibrotic markers (Fig. 6d). These findings indicate that periostin is required for the development of hepatic fibrosis induced by chronic hepatotoxicity.

Deletion of periostin in mice attenuates liver fibrosis caused by chronic cholestasis

We further investigated the influence of periostin deletion in a DDC feeding model in which the precipitation of protoporphyrins in the intrahepatic bile ducts leads to cholestatic liver injury and the development of hepatic fibrosis. Figure 7a shows that the addition of DDC to the diet resulted in severe hepatic fibrosis, with an enhanced ductular reaction being observed as cholangiocyte proliferation and formation of new intrahepatic bile ducts in wild-type mice. Furthermore, the serum levels of periostin

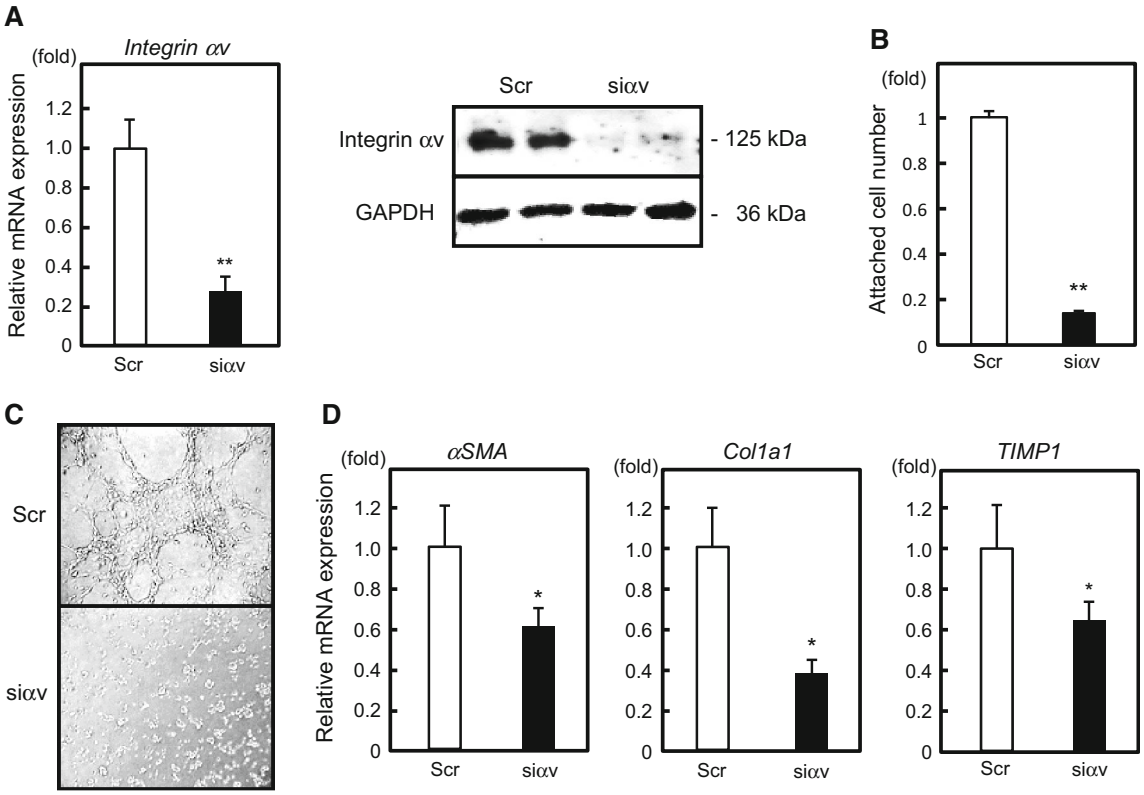


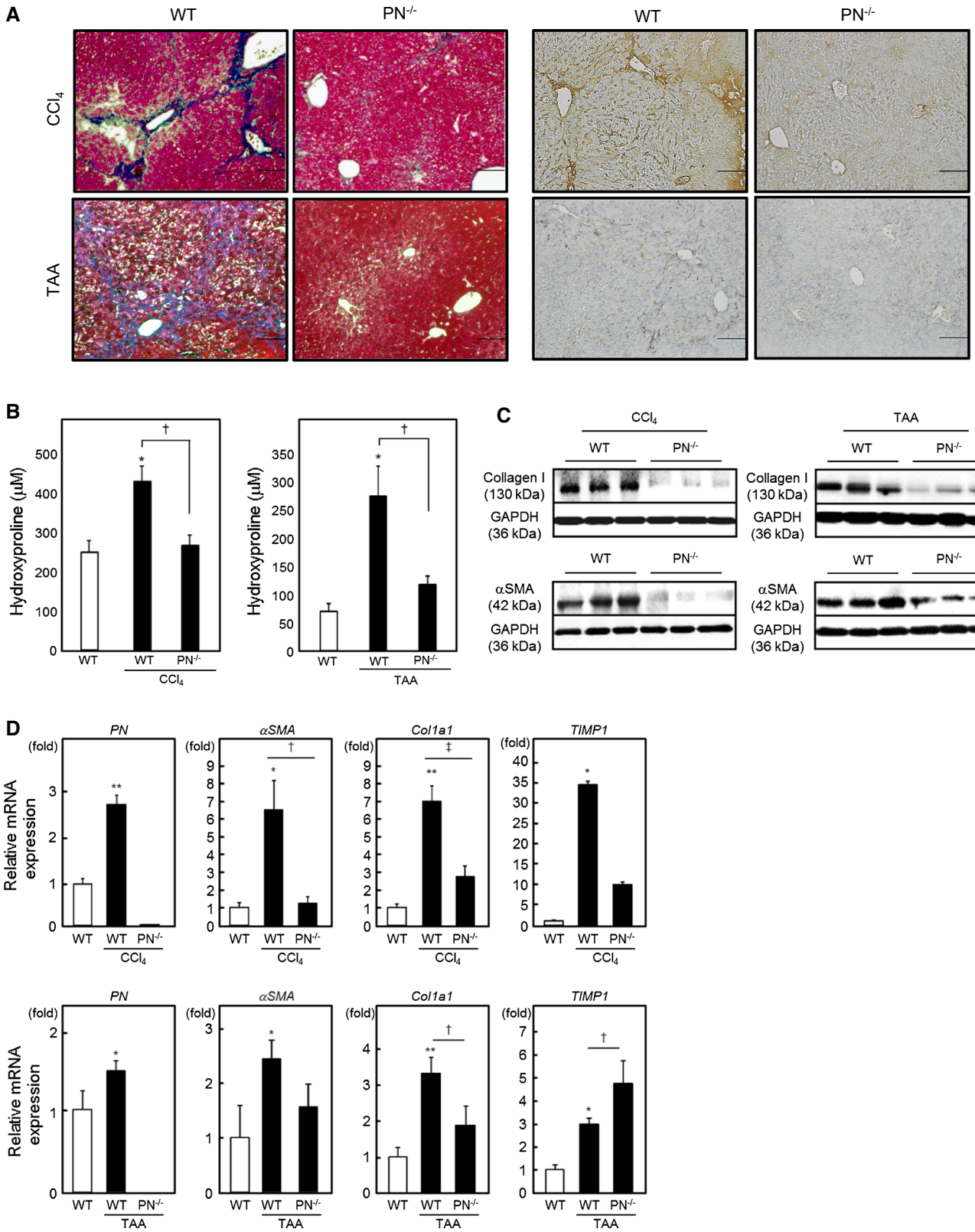
Fig. 5 Periostin activated hepatic stellate cells by interacting with α_v integrin. LX2 cells were transfected with either small interfering RNA (siRNA) targeting human α_v integrin (*siαv*) or nontargeting scramble control (*Scr*). **a** Knockdown of α_v integrin was confirmed by quantitative real-time polymerase chain reaction and immunoblot analysis. **b** A cell adhesion assay was performed with a periostin-coated plate on LX2 cells 72 h after transfection with siRNA. **c** Morphological characteristics of LX2 cells on periostin-coated plates with or without transfection with siRNA for α_v integrin (original magnification ×100). **d** Gene expression of profibrosis markers was quantified by quantitative real-time polymerase chain reaction ($n = 6$). Data are expressed as the mean ± standard error of the mean. *One asterisk* $P < 0.05$, *two asterisks* $P < 0.01$, *αSMA* α-smooth muscle actin, *Col1a1* collagen type I, alpha 1, *GAPDH* glyceraldehyde-3 phosphate dehydrogenase, *mRNA* messenger RNA, *TIMP1* tissue inhibitor of metalloproteinase 1

were dramatically elevated in DDC-fed mice compared with wild-type mice (148.88 ± 114.55 ng/mL vs 26.78 ± 25.20 ng/mL; $P < 0.05$). Immunostaining demonstrated an increase of α-SMA expression in wild-type mice together with periostin enhancement in the fibrotic septa around the proliferating ducts. Such changes were less prominent in the periostin$^{-/-}$ mice. These findings were supported by the quantification of the hepatic hydroxyproline content (Fig. 7b) and Western blot analysis of collagen type I (Fig. 7c). Quantitative real-time polymerase chain reaction demonstrated reduced expression of profibrotic genes, including *Col1a1* and *Timp1*, in periostin$^{-/-}$ mice (Fig. 7d). Furthermore, the expression of cytokeratin 19, a cholangiocyte marker, was increased in wild-type mice and less apparent in periostin$^{-/-}$ mice, supporting the histological difference of the ductular reactions (Fig. 7e). Taken together, these results strongly suggest that the deletion of periostin attenuates hepatic fibrosis and the ductular reaction induced by chronic cholestasis.

Discussion

Hepatic fibrosis is characterized by the excessive accumulation of ECM consisting of structural and nonstructural proteins. Despite not being structural components of the ECM, matricellular proteins have recently emerged as crucial regulators of cell–ECM interactions that modulate cell adhesion, migration, proliferation, differentiation, and apoptosis [29]. The current study provides novel evidence that periostin promotes the profibrotic properties of HSCs by interacting with α_v integrins, and that deletion of periostin in mice reduces hepatic fibrosis induced by chronic hepatotoxic injury as well as cholestatic injury.

Accumulating evidence suggests that various matricellular proteins regulate the biological functions of HSCs and the development of hepatic fibrosis. Osteopontin is one of the ECM molecules most strongly upregulated during liver injury, and it promotes HSC activation and collagen production [30]. Indeed, mice lacking osteopontin show less severe hepatic fibrosis after long-term treatment with CCl_4


A
WT
PN-/-
WT
PN-/-
CCl4
TAA
B
Hydroxyproline (μM)
WT
WT
PN-/-
CCl4
TAA
C
CCl4
TAA
Collagen I (130 kDa)
GAPDH (36 kDa)
αSMA (42 kDa)
D
PN
αSMA
Col1a1
TIMP1
Relative mRNA expression
(fold)

◀**Fig. 6** Deletion of periostin (*PN*) in mice attenuates hepatotoxic liver fibrosis. $PN^{-/-}$ and wild-type (*WT*) mice were subjected to either CCl_4 treatment twice weekly for 4 weeks or thioacetamide (*TAA*) administration in the drinking water for 16 weeks ($n = 5$–7). **a** Liver sections from WT and $PN^{-/-}$ mice were stained with azan staining or an antibody recognizing α-smooth muscle actin (*αSMA*; *dark brown*). *Scale bars* 100 μm. **b** The liver hydroxyproline content in WT and $PN^{-/-}$ mice was quantified. **c** Liver lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting with antibodies against αSMA and collagen type I. **d** The relative messenger RNA (*mRNA*) levels of fibrosis markers and endogenous PN were analyzed by quantitative real-time polymerase chain reaction. Data are expressed as the mean ± standard error of the mean. *One asterisk* $P < 0.05$, *two asterisks* $P < 0.01$, WT mice versus WT mice treated with CCl_4 or TAA, *dagger* $P < 0.05$, *double dagger* $P < 0.01$, WT mice treated with CCl_4 versus $PN^{-/-}$ mice treated with CCl_4, *Col1a1* collagen type I, alpha 1, *GAPDH* glyceraldehyde-3 phosphate dehydrogenase, *TIMP1* tissue inhibitor of metalloproteinase 1

or TAA [30, 31]. Likewise, deficiency of the ECM glycoprotein tenascin-C attenuates the development of hepatic fibrosis in mice with immune-mediated chronic hepatitis [32]. CCN2 (a matricellular protein of the CCN family; also known as "connective tissue growth factor") has also been shown to promote hepatic fibrosis synergistically with TGF-β [33, 34]. Collectively, these reports suggest that matricellular proteins play a pivotal role in hepatic fibrosis by modulating the functions of HSCs. In fact, our in vitro experiments demonstrated that the stimulation of periostin enhanced cell migration as well as activation of HSCs as shown in Fig. 3. Endogenous perisotin was also upregulated in response to perisotin, suggesting its role as a positive feedback loop. However, as demonstrated in Fig. S2c and d, upregulation of periostin in vivo did not reinduce α-SMA expression at both the messenger RNA level and the protein level. This might be explained by the possibility that the degree of induction of periostin was insufficient to lead to HSC reactivation in this experimental model. The future use of transgenic mice with liver-specific overexpression of periostin would provide new insights into this issue.

One of the highlights in the current study is that periostin-induced profibrotic properties of HSCs were mediated by α_v integrins as functional receptors. Integrins are transmembrane, heterodimeric proteins with noncovalently associated α and β subunits, which are involved in cell–cell and cell–ECM interactions [35]. It has been reported that integrins regulate many functions of HSCs, including cell proliferation, contraction, migration, and ECM synthesis [36, 37]. Previous studies have demonstrated that HSCs express $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_v\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_8$, $\alpha_6\beta_4$, and $\alpha_8\beta_1$ integrins. Of these, periostin interacts with $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_M\beta_2$, and $\alpha_6\beta_4$ integrins [37–40]. In the present study, the adhesion of LX2 cells to periostin was inhibited by antibodies targeting $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, identifying these α_v integrins as crucial receptors for periostin in HSCs. This was confirmed by the finding that cell adhesion was also dramatically reduced after α_v knockdown by siRNA. Importantly, the disruption of α_v integrin–periostin interactions significantly downregulated fibrotic gene expression (Fig. 5d), suggesting that this axis could be a potential target for the treatment of hepatic fibrosis. Consistent with our data, pharmacological blockade of α_v-containing integrins has been reported to attenuate liver fibrosis [40]. Furthermore, the inhibition of integrin signaling via the Arg–Gly–Asp (RGD) motif, which is the recognition sequence for many members of the integrin family, including $\alpha_v\beta_3$ and $\alpha_v\beta_5$, has been reported to disturb the activation of HSCs [37, 41].

The liver cell populations responsible for the secretion of periostin have not been well defined. Our in vitro experiments demonstrated that periostin expression in HSCs was increased in association with its activation. We also observed that the release of periostin from LX2 cells into the culture medium was increased in response to TGF-β, the most potent stimulator in HSC activation. These data suggest activated HSCs are a major source of periostin. However, periostin was barely detectable by immunohistochemistry in our models of hepatotoxic hepatic fibrosis (CCl_4 and TAA), although it was identified around proliferating bile ductules in our cholestatic fibrosis model (DDC). These findings suggest that periostin is rapidly secreted into the bile, so periostin immunostaining is seen only when the bile flow is disturbed. Consistent with this idea, the serum level of periostin in patients with biliary atresia was significantly higher than in controls [42]. In addition, immunostaining of liver sections from patients with noncholestatic liver cirrhosis has not detected periostin in either hepatocytes or fibrous hepatic stroma [42, 43]. In contrast, the overexpression of periostin in hepatic parenchymal cells has been reported in both human and experimental nonalcoholic fatty liver disease although periostin is preferentially secreted by tissue mesenchymal cells in other tissues [44, 45]. Collectively, the major source of periostin in the liver appears to depend on the cause of liver injury or fibrosis.

Another interesting finding of this study is that $periostin^{-/-}$ mice that were fed DDC demonstrated a dramatically less marked ductular reaction, characterized by bile duct expansion and proliferation together with lower expression of cytokeratin 19, indicating that periostin is also required for this process to occur. This finding is supported by a report that the blockade of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins by cilengitide, a cyclic RGD pentapeptide, suppresses the ductular reaction after bile duct ligation [40]. Similar observations have been made in mice lacking other matricellular proteins, including osteopontin, CCN2, and

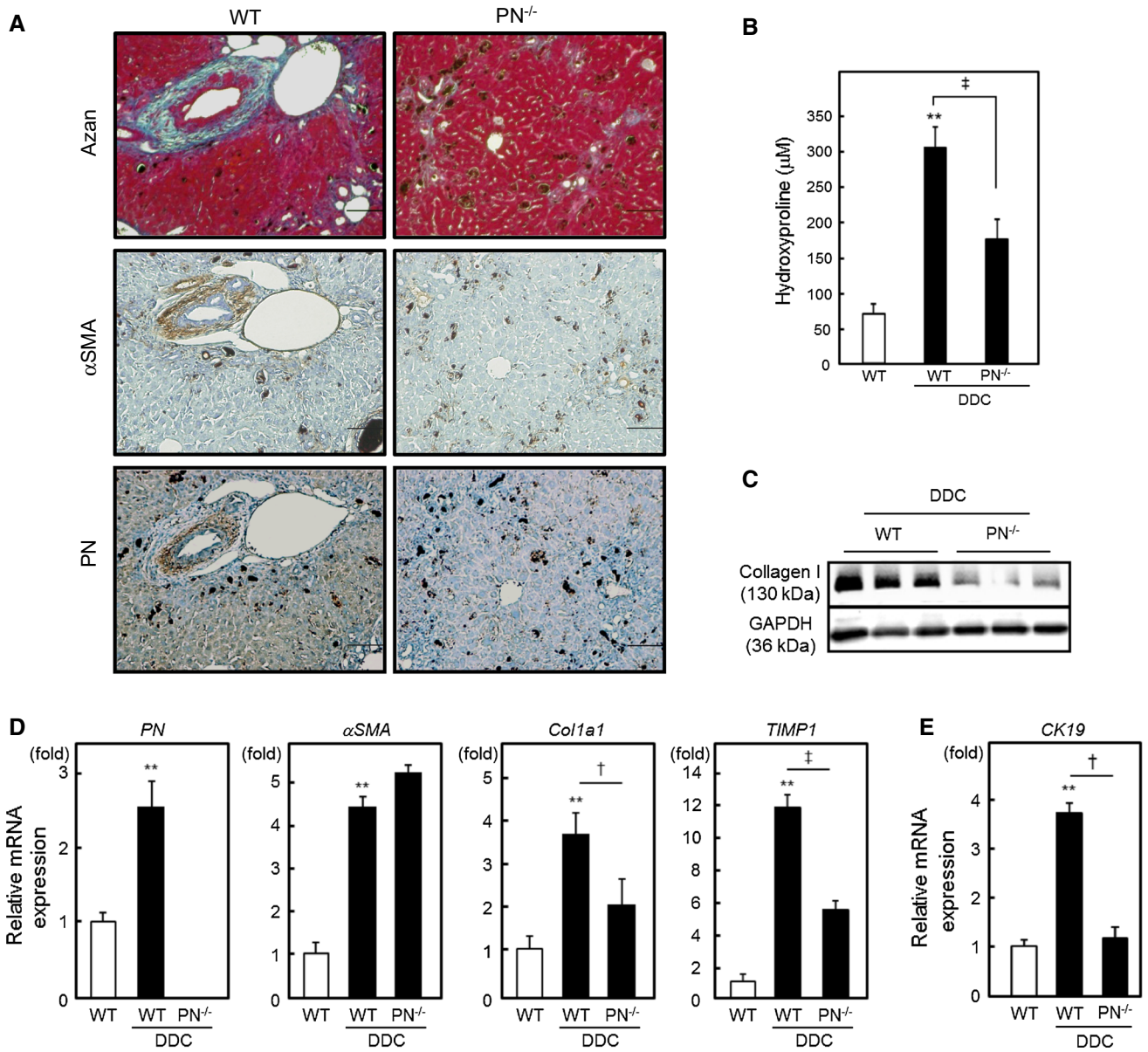


Fig. 7 Deletion of periostin (*PN*) in mice attenuates cholestatic liver fibrosis. $PN^{-/-}$ mice and wild-type (*WT*) mice were fed a 0.1 % 3,5-diethoxycarbonyl-1,4-dihydrocollidine (*DDC*)-containing diet for 4 weeks ($n = 6$–7). **a** Liver sections from WT and $PN^{-/-}$ mice were stained with azan staining or antibody recognizing α-smooth muscle actin (*αSMA*; *dark brown*). *Scale bars* 100 μm. **b** The liver hydroxyproline content in WT and $PN^{-/-}$ mice was quantified. **c** Liver lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting with an antibody against collagen type I. **d**, **e** Relative messnger RNA (*mRNA*) levels of fibrosis markers, endogenous PN, and cytokeratin 19 (*CK19*) were analyzed by quantitative real-time polymerase chain reaction. Data are expressed as the mean ± standard error of the mean. *One asterisk* $P < 0.05$, *two asterisks* $P < 0.01$, WT mice versus WT mice fed DDC, *dagger* $P < 0.05$, *double dagger* $P < 0.01$, WT mice fed DDC versus $PN^{-/-}$ mice fed DDC, *Col1a1* collagen type I, alpha 1, *GAPDH* glyceraldehyde-3 phosphate dehydrogenase, *TIMP1* tissue inhibitor of metalloproteinase 1

CCN1, all of which bind to $\alpha_v\beta_3$ integrin [31, 33, 45]. Taken together, these results suggest that $\alpha_v\beta_3$ integrin signaling is critical for ductular reaction.

There have recently been reports demonstrating that the deletion of periostin in mice reduces the development of experimental hepatic fibrosis induced by a diet deficient in methionine and choline or by treatment with CCl_4 [20, 21]. In addition to supporting these previous data obtained in CCl_4 model, the present study further explored the significant role of periostin in hepatic fibrosis as observed in the cholestatic DDC model. To our knowledge, this is the first report demonstrating periostin is an indispensable regulator

for biliary fibrosis. Furthermore, the TAA model has an advantage over the CCl_4 model in terms of hepatic histological changes resembling human cirrhosis [46].

In conclusion, the biological function of HSCs, including cell motility, collagen synthesis, and endogenous periostin induction, have been proved to be regulated by periostin–α_v integrin interaction. On the basis of our cumulative data, the modulation of this interaction is a potential approach for the treatment of hepatic fibrosis.

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Conflict of interest Kenji Izuhara received a research grant from Chugai Pharmaceutical Co. Ltd and unrestricted grant from Shino-test Co. Ltd, and serves as a consultant to Chugai Pharmaceutical Co. Ltd. and AQUA Therapeutics Co. Ltd.

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