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

--Manuscript Draft--

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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 HSC-immortalized cell line LX2 were utilized to study the profibrotic property of periostin and the interaction of periostin with integrins. Wild-type (WT) 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 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 αvβ5 and αvβ3 integrins suppressed cell attachment to periostin by 60% and 30%, respectively, while anti-α5β1 antibody had no effect. Consistently, αv integrin-silenced LX2 exhibited decreased cell attachment to periostin with a significant reduction in profibrotic markers. Moreover, these profibrotic effects of periostin were observed in the mouse models. In contrast to extensive collagen deposition in WT 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. Conclusions: Periostin exerts potent profibrotic activity mediated by αv integrin, suggesting the periostin-αv integrin axis as a novel therapeutic target for hepatic fibrosis.
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Author Comments:	March 9, 2016 Prof. Akira Andoh Editor-in-Chief Journal of Gastroenterology RE: JOGA-D-16-00021 Dear Prof. Andoh: Attached please find a revised manuscript (JOGA-D-16-00021) entitled "Periostin promotes hepatic fibrosis in mice by modulating hepatic stellate cell activation via αv integrin interaction" by Akiko Sugiyama et al, which we would be grateful to have considered for publication in Journal of Gastroenterology. We appreciate the constructive comments from the editor and the expert reviewers, and have included a point-by-point reply. Based on the critical feedback, we have performed additional experiments and incorporated the results into the manuscript, which has undergone major revisions. All necessary changes made to the text have been highlighted for convenience. This work has not been previously published or reported, and this manuscript is not under consideration elsewhere, nor will it be submitted elsewhere while under review by Journal of Gastroenterology. We hope that our manuscript is now acceptable for publication. Sincerely yours, Keishi Kanno, MD, PhD.
Response to Reviewers:	Point-by-point response Reviewer #1 1. This revised manuscript has been improved by responding to the comments by reviewer. Regarding the results of alpha-SMA after 14 days of single injection of CCl4 in supplementary Fig.2, authors are advised to discuss about the unexpected results in Discussion section, as described in author's response to reviewer comments. We appreciate the Reviewer’s suggestion, and this issue has now been discussed in the revised manuscript.

March 25, 2016

Prof. Akira Andoh
Editor-in-Chief
Journal of Gastroenterology

RE: JOGA-D-16-00021R1

Dear Prof. Andoh:

Attached please find a revised manuscript (JOGA-D-16-00021R1) entitled “Periostin promotes hepatic fibrosis in mice by modulating hepatic stellate cell activation via αv integrin interaction” by Akiko Sugiyama et al, which we would be grateful to have considered for publication in Journal of Gastroenterology.

We appreciate that both reviewers considered the work to be valuable. According to the suggestion from the Reviewer #1, we have revised the discussion section in the manuscript.

This work has not been previously published or reported, and this manuscript is not under consideration elsewhere, nor will it be submitted elsewhere while under review by Journal of Gastroenterology.

We hope that our manuscript is now acceptable for publication.

Sincerely yours,

Keishi Kanno, MD, PhD.

Point-by-point response

Reviewer #1

1. This revised manuscript has been improved by responding to the comments by reviewer. Regarding the results of alpha-SMA after 14 days of single injection of CCl4 in supplementary Fig.2, authors are advised to discuss about the unexpected results in Discussion section, as described in author's response to reviewer comments.

We appreciate the Reviewer’s suggestion, and this issue has now been discussed in the revised manuscript.

Title

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

Authors

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Role of periostin in hepatic fibrosis

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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 HSC-immortalized cell line LX2 were utilized to study the profibrotic property of periostin and the interaction of periostin with integrins. Wild-type (WT) 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 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, while anti-$\alpha 5\beta 1$ antibody had no effect. Consistently, αv integrin-silenced LX2 exhibited decreased cell attachment to periostin with a significant reduction in profibrotic markers. Moreover, these profibrotic effects of periostin were observed in the mouse models. In contrast to

extensive collagen deposition in WT 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 αv integrin, suggesting the periostin-αv integrin axis as a novel therapeutic target for hepatic fibrosis.

Keywords

matricellular protein, liver fibrogenesis, cell adhesion receptor, myofibroblasts, cell migration.

List of Abbreviations

α-SMA, α-smooth muscle actin; BDL, bile duct ligation; BSA, bovine serum albumin; CCl_4, carbon tetrachloride; CK, cytokeratin; Col1a1, collagen1a1; CTGF, connective tissue growth factor; DDC, 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine; DOC, deoxycholic acid; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSCs, hepatic stellate cells; IL-6, interleukin-6; NAFLD, non-alcoholic fatty liver disease; PFs, portal fibroblasts; PLL, poly-L-lysine; RIPA, radioimmunoprecipitation assay buffer; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TAA, thioacetamide; TIMP-1, tissue inhibitor of metalloproteinase 1; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor- α, WT, wild-type

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 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 [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 morbidity and mortality worldwide [13]. In an attempt to develop effective anti-fibrosis 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 (PFs), depending on the etiology of liver injury [14]. Activated HSCs are widely recognized as the main cells contributing to liver fibrosis associated with hepatotoxicity. Although activated PFs are implicated in the early stages of cholestatic fibrosis induced by bile duct ligation (BDL), the contribution of PFs decreases with the progression of liver damage, and HSCs predominate, suggesting that HSCs are the chief contributors to hepatic fibrosis, independent of its etiology [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 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)-β1 *in vitro* [19]. In addition, periostin$^{-/-}$ mice exhibited resistance to experimental liver fibrosis induced by either CCl_4 or methionine choline deficient diet [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 and cholestatic injury.

Materials and Methods

Animal Models of Liver Fibrosis

Male periostin$^{-/-}$ mice (C57BL/6 background, 8–10 weeks) 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 (IP) injection of 25% solution of carbon tetrachloride (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 hepatotoxic models, mice were treated by IP 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 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, immortalized human activated HSCs (a gift from Scott L. Friedman, Mount Sinai Hospital, New York, NY, USA) were cultured in Dulbecco's modified Eagle 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 siRNA

Small interfering RNAs (siRNAs) specific to human periostin, αv integrin (Invitrogen, Grand island, NY, USA), and a non-silencing negative control (Sigma-Aldrich Japan, Hokkaido, Japan) were transfected using 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 αv integrin, 5'-GGUCCAAGUUCAUUCAGCAAGGCAA-3' and 5'-UUGCCUUGCUGA AUGAACUUGGACC-3'. To investigate whether silencing of periostin induces HSCs apoptosis, immunoassay for single-stranded DNA in HSCs following siRNA transfection was performed using ApoStrand™ ELIZA apoptosis detection kit (Enzo Life Sciences, Inc. Farmingdale, NY, USA).

Flow Cytometry

Flow cytometric analysis for the expression of αvβ3, α5β1, and αvβ5 integrins was performed using primary antibodies against integrin αvβ3 (LM609; Merck Millipore, Tullagreen, Ireland), α5 (JBS5; Abcam, Cambridge, England), and αvβ5 (ALULA; a gift from Dean Sheppard, UCSF, USA), with goat anti-mouse PE (Santa Cruz, Dallas, TX, USA) as a secondary antibody. Expression profiles were acquired using a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

Scratch Assay

Confluent cultures of LX2 incubated for 24 h in a serum-free medium were scratched using a sterile pipette tip, and were incubated either with phosphate buffered saline (PBS) or 5 μg/mL recombinant human periostin/OSF-2 (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 Inc., Corning, NY, USA) were coated with 5 μg/mL recombinant human periostin or PBS. 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. Migrated cells 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 [25], with slight modifications. Briefly, 96 wells of MaxiSorp NUNC-Immuno Plate (Thermo Scientific, Waltham, MA, USA) were coated with 5 μg/mL of recombinant human periostin or 10 μg/mL 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 (BSA; Nacalai Tesque, Kyoto, Japan) in DMEM, plates were filled with 50 μL of LX2 cell suspension (10 × 10^5 cell/mL in DMEM) with or without neutralizing antibodies, and centrifuged at 1000 x*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 x*g* for 5 min, followed by cell counting.

Hydroxyproline Assay

Liver tissues (30 mg/300 μL in sterilized water) were hydrolyzed with 6N HCl for 3 h at 120°C. The

precipitates were removed by a MILLEX-HV 0.45 μm filter unit (Merck Millipore, Darmstadt, Germany), the hydroxyproline concentration was quantified using a Hydroxyproline Colorimetric Assay Kit (BioVision, Milpitas, CA, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed as described previously with β-actin as an internal control [26]. The sequences of specific primers were listed in Supplementary Table 1.

Western Blot Analysis

Cells and liver tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer [0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid (DOC), 1% NP-40, 150 mM NaCl, and 50 mM Tris-Cl, pH 7.6] and centrifuged at 10000 x*g* for 10 min. The supernatant was subjected to Western blot analysis using the primary antibodies against α-smooth muscle actin (α-SMA; ab15734, Abcam, Cambridge, England; A5228, Sigma-Aldrich, St. Louis, MO, USA), periostin (ab14041, Abcam; ab92460, Abcam), collagen type I (ab 59435, ab34710; Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 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 or azan staining. Immunohistochemistry was performed using the antibody against periostin (ab14041; Abcam) or α-SMA.

Analytical Techniques

Plasma periostin levels were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using originally developed anti-periostin monoclonal antibody (clone no. SS19D) [27]. Concentrations of periostin in the conditioned media were measured using a human periostin ELISA Kit (Shino-Test Co., Tokyo, Japan). Plasma concentrations of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were enzymatically determined.

Statistical Analysis

Data were analyzed by two-way ANOVA or two-sided, unpaired Student's *t* tests. Results are expressed as mean ± standard error of the mean (SEM). 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 BDL and studied the expression of periostin in the fibrotic livers. In the homogenates of the livers from the two models, both the gene and protein expressions of periostin were significantly elevated (Supplemental Fig. 1A and B). The serum level of periostin was also significantly higher in CCl_4-treated mice compared with 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 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 AST and ALT (Supplemental Fig. 2A and B). Periostin gene expression peaked at day 3, in the same manner as collagen1a1 (Col1a1). In contrast, the expression of α-SMA, a marker of HSC activation, peaked as early as day 1 after the administration along with inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF) α. The protein levels of periostin also became most elevated after that of αSMA reached most abundant (Supplemental Fig. 2C and D). These results suggest that similar to collagen1, 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-β1 [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-β1. Following TGF-β1 stimulation, periostin gene expression gradually increased up to about 5-fold 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 and C). These changes were parallel to the increase in α-SMA. With the plate activation of rat primary HSCs, an increase in periostin expression was also observed, as with α-SMA and Col1a1 (Fig. 1D and 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 re-capitulates many features of activated HSC phenotype, we performed knockdown using siRNA. Periostin mRNA 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, Col1a1, and tissue inhibitor of metalloproteinase 1 (TIMP-1), was also significantly downregulated (Fig. 2B).

Periostin knockdown did not induce HSCs apoptosis, suggesting the possibility that targeted deletion of endogenous periostin deactivated HSCs by interfering with its autocrine loop (Fig. 2C). As previous studies have indicated that periostin acts downstream of TGF-β [2], we investigated the influence of TGF-β1 on LX2 cells, with or without periostin disruption. Although TGF-β1 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-β1.

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. Fig. 3A shows the enhanced non-directional migration of HSCs into the scratch area after stimulation with recombinant periostin. In the Transwell migration assay, coating the underside of the chamber membrane with recombinant periostin led to a two-fold 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 I) and endogenous periostin as compared to PLL, 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 αv *Integrin*

As periostin is a ligand for integrins, which are reported to play a role in collagen synthesis by HSCs [17], we 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-α5β1 antibody or control IgG (Fig. 4C). The release of collagen into the culture medium was also reduced by anti-integrin antibodies (data not shown). These results suggest critical role for αv integrin in the activation of HSCs.

Periostin Activates HSCs by Interacting with αv Integrin

To further assess the interaction of periostin with the αv integrin, LX2 cells were transduced with siRNAs targeting αv integrin, and successful knockdown was achieved (Fig. 5A). As shown in Fig. 5B, the knockdown of αv integrin dramatically inhibited the attachment of LX2 cells to periostin by approximately 90%, as assessed by the cell adhesion assay. The morphological changes of LX2 cells with αv knockdown on periostin-coated plates (Fig. 5C) were similar to those observed following incubation with neutralizing antibodies against αv integrins (Fig. 4C). Furthermore, profibrotic markers, α-SMA, Col1a1, and TIMP-1, were significantly reduced by the disruption of αv integrin, suggesting that the interaction between periostin and αv integrin is critical for the activation of HSCs.

Deletion of Periostin in Mice Reduces Susceptibility to Hepatotoxic Liver Fibrosis

Based on 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. Fig. 6A displays representative histological findings in the livers of wild-type (WT) and periostin$^{-/-}$ mice after the administration of either CCl_4 or TAA. In both models of chronic hepatotoxicity, WT mice exhibited extensive collagen deposition in the liver, with an increase in activated HSCs expressing α-SMA mainly in the fibrotic 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 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 I and α-SMA proteins (Fig. 6C). Quantitative PCR 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. Fig. 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 WT mice. Furthermore, the serum levels of periostin were dramatically elevated in DDC-fed mice compared with WT 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 WT 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 I (Fig. 7C). Quantitative RT-PCR demonstrated reduced expression of profibrotic genes, including *Col1a1* and *TIMP-1*, in periostin$^{-/-}$ mice (Fig. 7D). Furthermore, the expression of cytokeratin (CK) 19, a cholangiocyte marker, was increased in WT 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 as 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 αv integrins, and that deletion of periostin in mice reduces hepatic fibrosis induced by chronic hepatotoxic injury as well as cholestatic injury.

Accumulating evidences 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 chronic treatment with CCl_4 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, CTGF) 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 of positive feedback loop. However, as demonstrated in supplemental Fig. 2C and D, upregulation of periostin *in vivo* did not reinduce α-SMA expression at both mRNA and protein levels. This might be explained by the possibility that the induction of periostin was insufficient in quantity to lead to HSCs reactivation in this experimental model. The future use of transgenic mice with liver-specific overexpression of periostin would provide new insights on this issue.

One of the highlights in the current study is that preriostin-induced profibrotic properties of HSCs was mediated by αv 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 α1β1, α2β1, αvβ1, α5β1, αvβ3, αvβ5, αvβ8, α6β4, and α8β1 integrins. Of these, periostin interacts with αvβ1, αvβ3, αvβ5, αMβ2, and α6β4 integrins [37-40]. In the present study, the adhesion of LX2 cells to periostin was inhibited by antibodies targeting αvβ3 and αvβ5 integrins, identifying these αv integrins as crucial receptors for periostin in

HSCs. This was confirmed by the finding that cell adhesion was also dramatically reduced after αv knockdown by siRNA. Importantly, the disruption of αv 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 αv-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 αvβ3 and αvβ5, 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 HSC was increased in association with its activation. We also observed that the release of periostin from LX2 into the culture medium was increased in response to TGF-β, the most potent stimulator in HSC activation. These data suggest activated HSC as 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 may suggest that periostin is rapidly secreted into the bile, so periostin immunostaining is only seen when the bile flow is disturbed. Consistent with this idea, the serum

level of periostin in patients with biliary atresia was significantly higher than control [42]. In addition, immunostaining of liver sections from patients with non-cholestatic 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 non-alcoholic 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 etiology of liver injury or fibrosis.

Another interesting finding of this study was 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 CK19, 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 BDL [40]. Similar observations have been made in mice lacking other matricellular proteins, including osteopontin, CCN2, and 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 methionine-choline deficient

diet or treatment of CCl_4 [20,21]. In addition to supporting these previous data shown in CCl_4 model, the present study further explored the significant role for periostin in hepatic fibrosis as observed in cholestatic DDC modell. To our knowledge, this is the first report demonstrating periostin as an indispensable regulator for biliary fibrosis. Furthermore, TAA model has an advantage over CCl_4 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 are proved to be regulated by periostin-αv integrin interaction. Based upon our cumulative data, the modulation of this interaction is a potential approach for the treatment of hepatic fibrosis.

Author Contributions

A.S. and K.K. prepared the draft of the manuscript. A.S., K.K., S.O., J.O., and N.N. performed the experiments. A.S., K.K., and S.T. contributed to the study concept and design. Y.Y., S.C, and K.I. helped with the material support and interpretation of data.

Acknowledgments

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Figure legends

Fig. 1. Periostin induction is associated with HSCs activation in vitro

(A) A human HSC line, LX2, was stimulated with 10 ng/mL of TGF-β or vehicle (Veh), and periostin (PN) transcripts were quantified by qRT-PCR (n = 4) at the indicated time points. (B) Protein expression of PN was analyzed by Western blot analysis (TGF-β: 10 ng/mL, 72 h). (C) PN concentrations of cultured media in which LX2 cells were stimulated by TGF-β (10 ng/mL, 72 h) were measured by ELISA. (D and E) Rat primary HSCs were activated by culturing on a plastic dish and PN expression was analyzed by qRT-PCR (n = 4) and immunoblotting. Data are expressed as mean ± SE. **P* < 0.05; ***P* < 0.01, vs. Veh on day 5.

Fig. 2. Periostin knockdown in HSCs reduces fibrosis markers

LX2 cells were transfected with either siRNA targeting human periostin (siPN) or non-targeting scramble control (Scr). (A) Knockdown of PN was confirmed by qRT-PCR (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 qRT-PCR 120 h after siRNA transfection. (C) Single-stranded DNA in LX2 after 120 hr of siRNA transfection was quantified by ELISA kit (n=8). (D) LX2 cells transfected with either siPN (black bar) or non-targeting control RNA (white bar) were treated with

TGF-β (10 ng/mL, 48 h), and the mRNA level of α-SMA was quantified by qRT-PCR (n = 6). Data are expressed as mean ± SE. *P < 0.05; **P < 0.01.

Fig. 3. Periostin enhances HSC 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 using a 200 μL pipet chip. Cells were cultured with recombinant periostin (PN) (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 recombinant PN (5 μg/mL) or PBS. The LX2 cells were allowed to migrate to the lower chambers containing 1% FBS DMEM for 24 h, followed by cell counting on the underside of the membrane at six randomly selected areas. (C) LX2 cells were plated on recombinant PN (0.5–10 μg/ mL), poly-L-lysine (PLL) or BSA (N/C: negative control)-coated plates and incubated for 1 h. After the 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 recombinant PN (5 μg/ mL) or a PLL-coated plate and incubated for 48 h. Immunoblotting was performed for the detection of α-SMA, collagen I and endogenous PN. Data are expressed as mean ± SE. *P < 0.05; **P < 0.01, vs. control.

Fig. 4. Periostin interacts with αv integrin

(A) Surface expressions of αvβ3, α5β1, and αvβ5 integrins on LX2 were analyzed by flow cytometry. (B) LX2 cells were plated on plates coated with 5 μg/mL of recombinant periostin (PN) in the presence or absence of a neutralizing antibody against αvβ3, α5β1 or αvβ5 integrin, or in a combination, and incubated for 2 h. After the 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 mean ± SE. *$P < 0.05$, vs. PN.

Fig. 5. Periostin activated HSCs via interacting αv integrin

LX2 cells were transfected with either siRNA targeting human αv integrin (siαv) or non-targeting scramble control (Scr). (A) Knockdown of αv integrin was confirmed by qRT-PCR and immunoblot analysis. (B) Cell adhesion assay was performed using a periostin (PN)-coated plate on LX2 72 h after transfection with siRNA. (C) Morphological characteristics of LX2 on PN-coated plates with or without transfection with siRNA for αv integrin (original magnification 100×). (D) Gene expression of

profibrosis markers was quantified by qRT-PCR (n = 6). Data are expressed as mean ± SE. *P < 0.05; **P < 0.01.

Fig. 6. Deletion of periostin in mice attenuates hepatotoxic liver fibrosis

Periostin $(PN)^{-/-}$ and WT mice were subjected to either CCl_4 treatment twice weekly for 4 weeks or 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-SMA (*dark brown*). Scale bars: 100 μm. (B) The liver hydroxyproline content in WT and $PN^{-/-}$ mice was quantified. (C) Liver lysates were resolved on SDS-PAGE followed by Western blotting using antibodies against α-SMA and collagen I. (D) The relative mRNA levels of fibrosis markers and endogenous PN were analyzed by qRT-PCR. Data are expressed as mean ± SE. *P < 0.05; **P < 0.01, WT vs. WT on CCl_4 or TAA, $^{\dagger}P$ < 0.05; $^{\ddagger}P$ < 0.01, WT on CCl_4 vs. $PN^{-/-}$ on CCl_4.

Fig. 7. Deletion of periostin in mice attenuates cholestatic liver fibrosis

Periostin $(PN)^{-/-}$ mice and WT mice were fed a 0.1% 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–SMA (*dark brown*). Scale bars: 100 μm. (B) The liver hydroxyproline content in WT and $PN^{-/-}$

mice was quantified. (C) Liver lysates were resolved on SDS-PAGE followed by Western blotting using an antibody against of collagen I. (D and E) Relative mRNA levels of fibrosis markers, endogenous PN, and CK19 were analyzed by qRT-PCR. Data are expressed as mean ± SE. *P < 0.05; **P < 0.01, WT vs. WT on DDC, †P < 0.05; ‡P < 0.01, WT on DDC vs. $PN^{-/-}$ on DDC.

Figure

Figure 1

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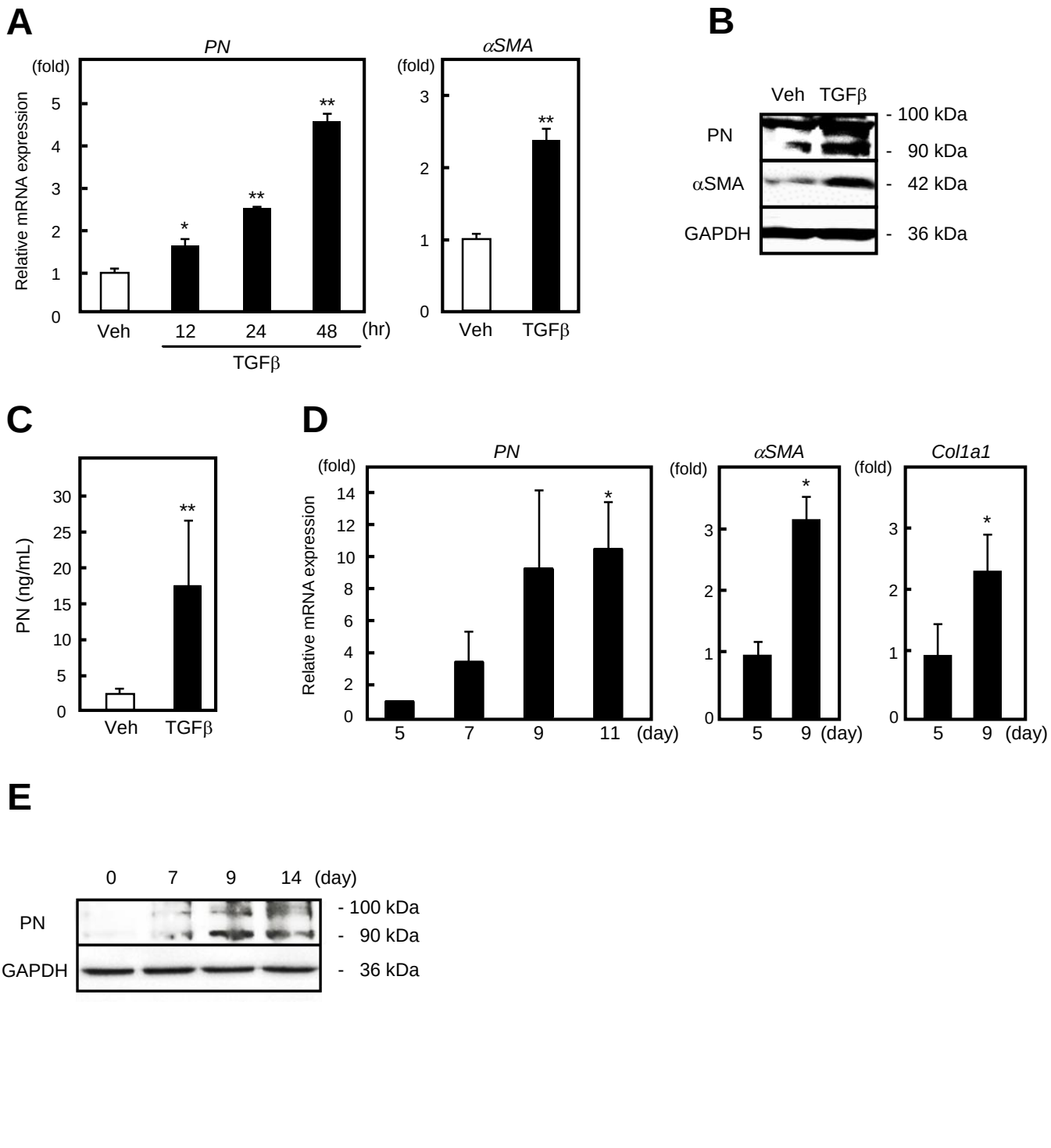
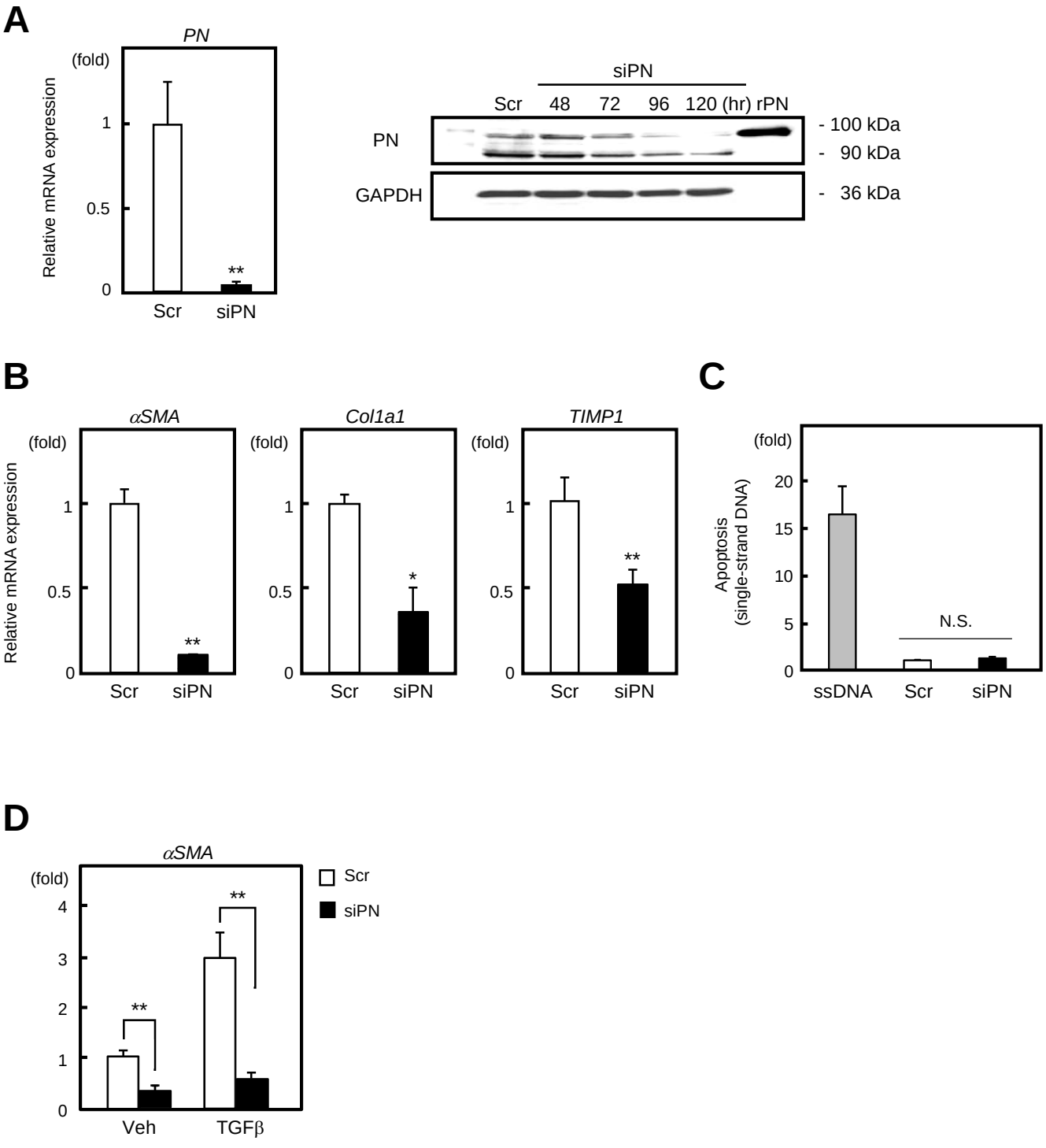


Figure 2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siPN
siPN
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GAPDH
- 100 kDa
- 90 kDa
- 36 kDa
B
αSMA
Col1a1
TIMP1
(fold)
Relative mRNA expression
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0.5
0
**
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C
(fold)
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Scr
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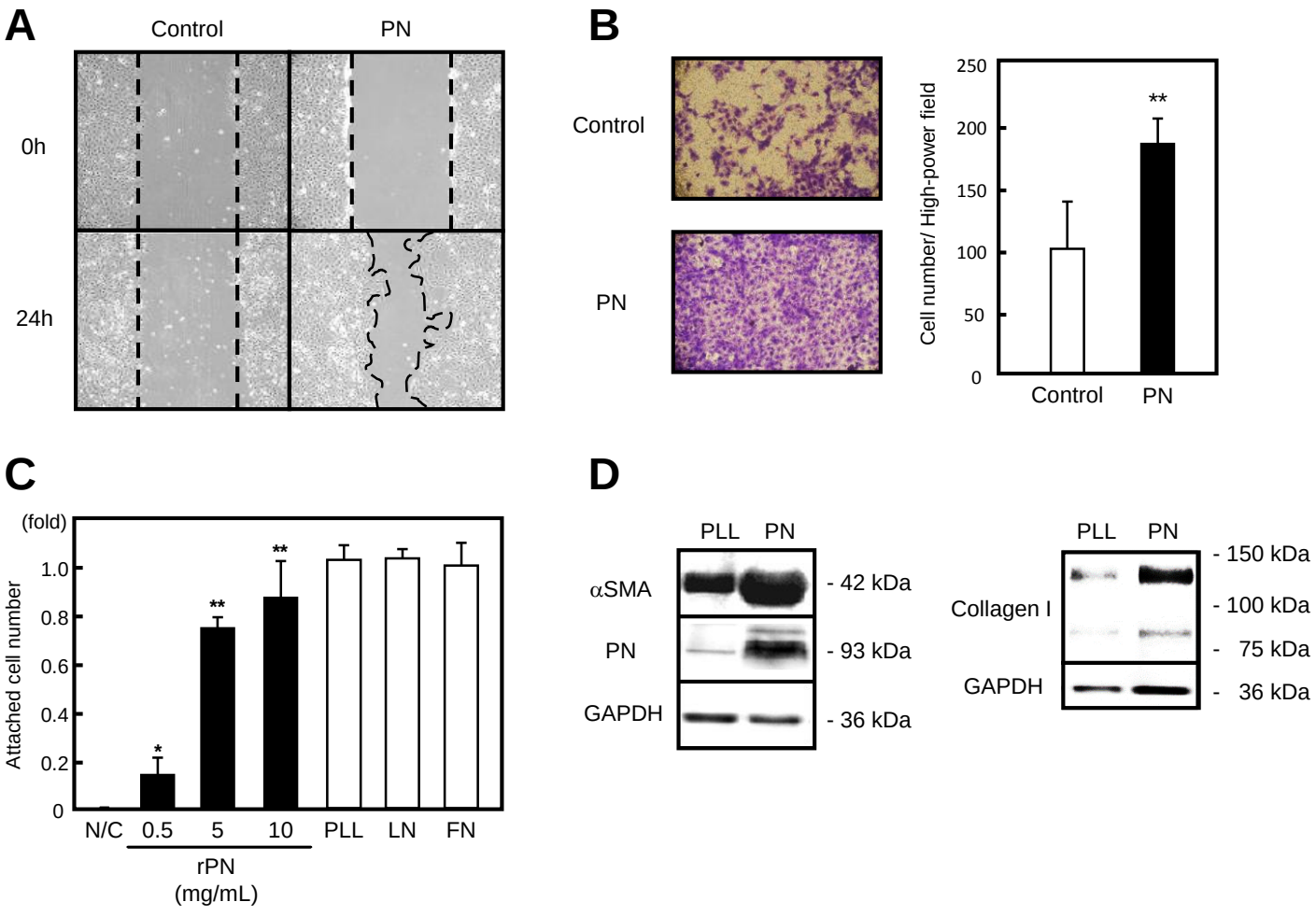
Veh
TGFβ

Figure 3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PN
0h
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Control
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Cell number/ High-power field
250
200
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100
50
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C
(fold)
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1.0
0.8
0.6
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10
PLL
LN
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(mg/mL)
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αSMA
PN
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- 100 kDa
- 75 kDa
- 36 kDa

Figure 4

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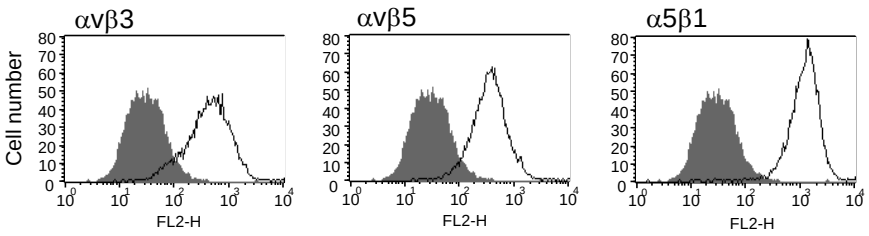
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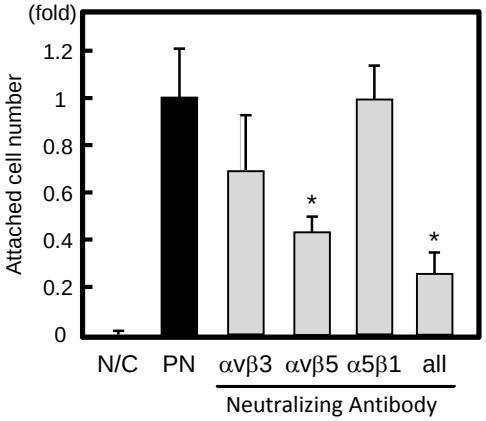
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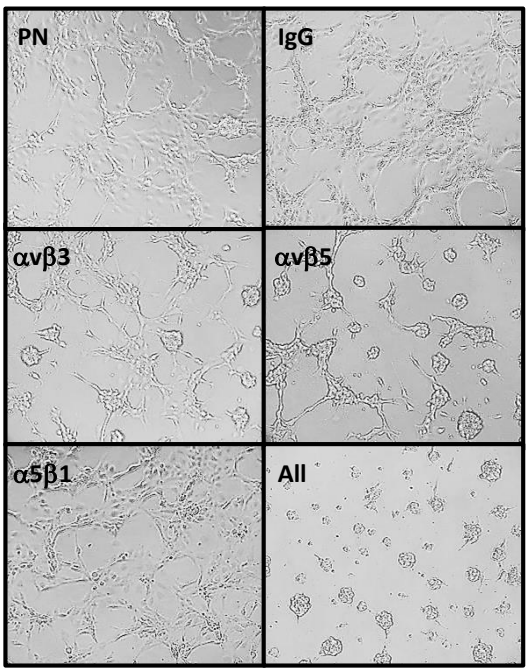
PN
αvβ3
αvβ5
α5β1
all
Neutralizing Antibody

C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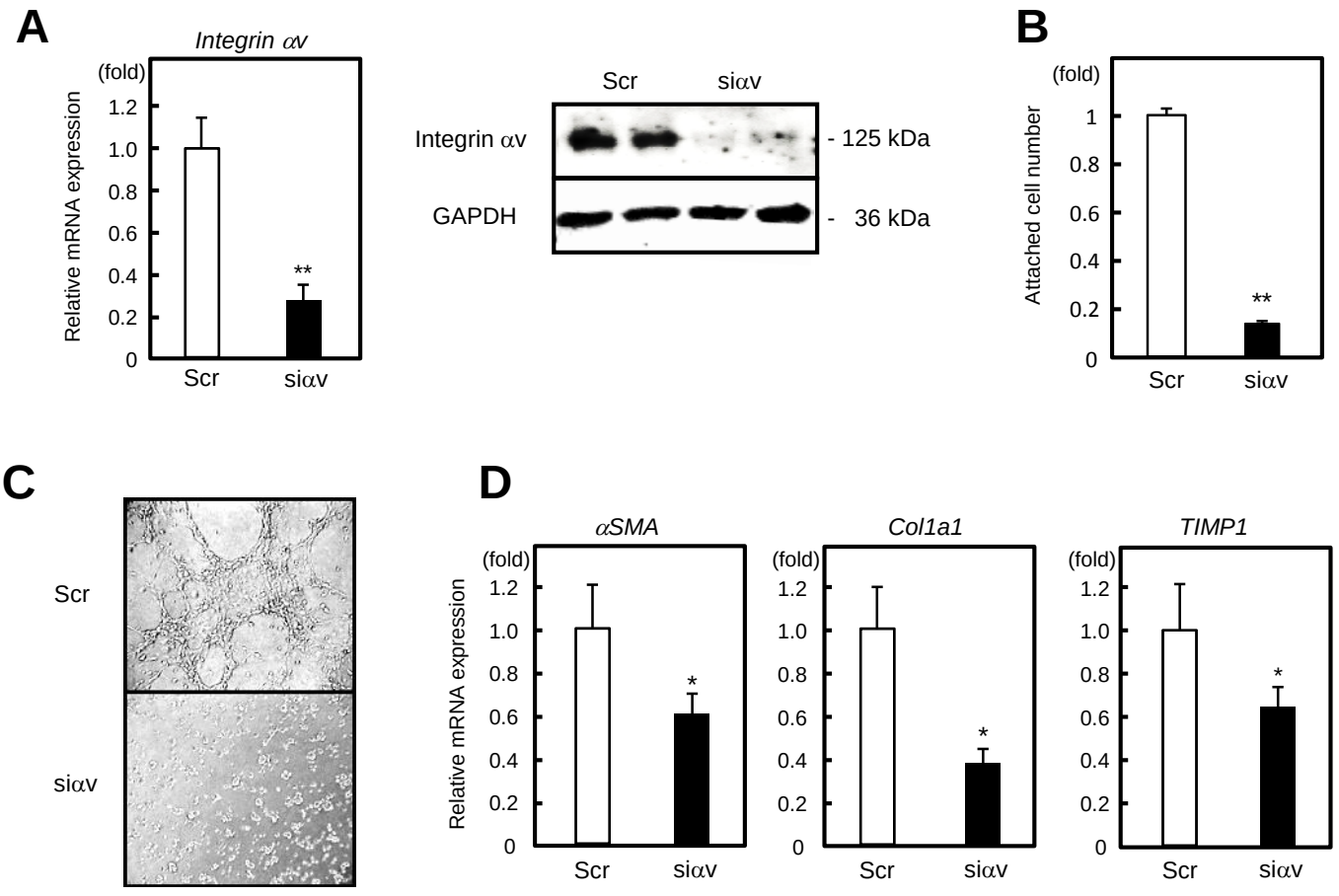
α5β1
All

Figure 5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Integrin αv
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0.8
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siαv
Scr
siαv
Integrin αv
- 125 kDa
GAPDH
- 36 kDa
B
(fold)
Attached cell number
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0.8
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0.4
0.2
0
**
Scr
siαv
C
Scr
siαv
D
αSMA
Col1a1
TIMP1
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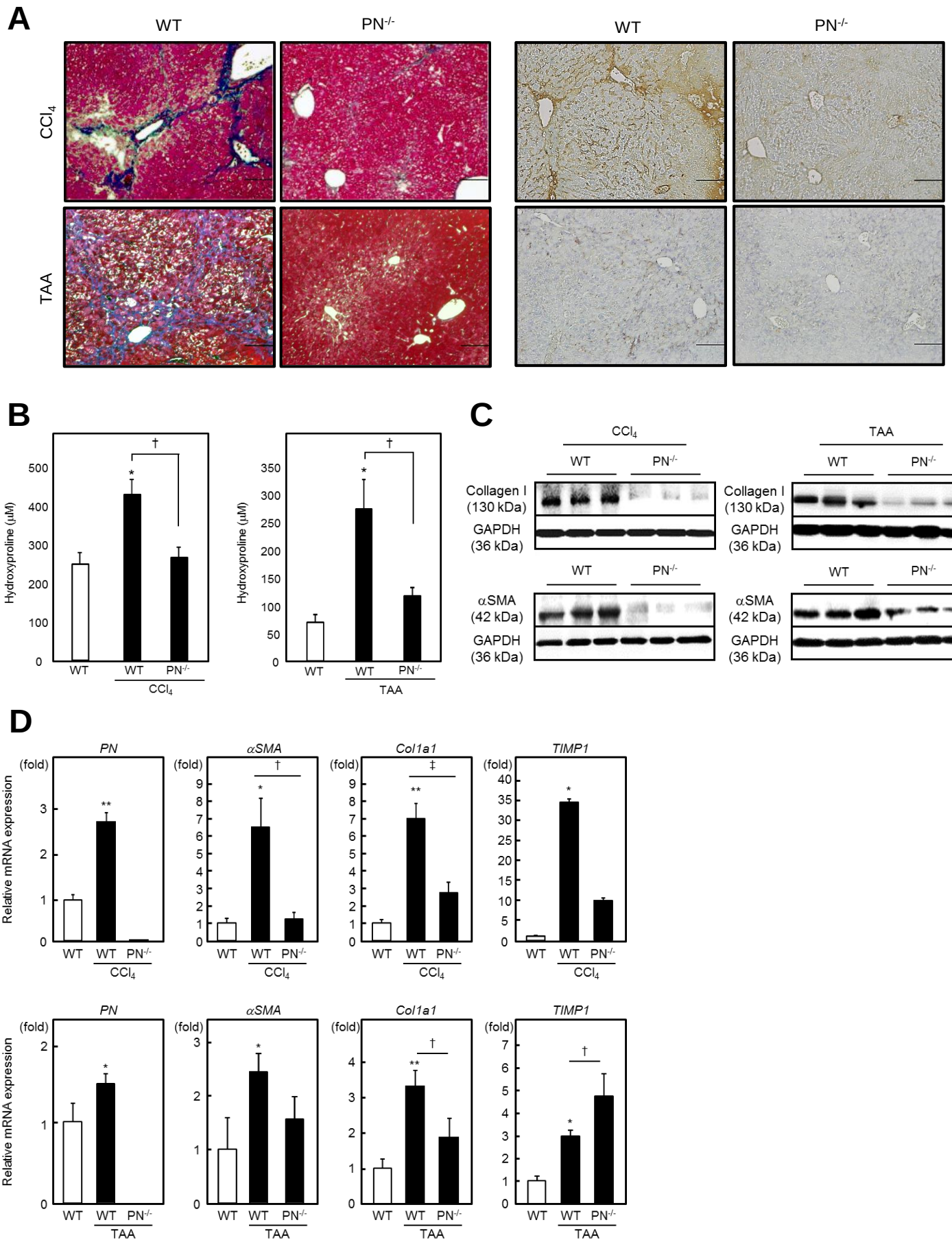
*
Scr
siαv

Figure 6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WT
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TAA
C
CCl4
TAA
Collagen I (130 kDa)
GAPDH (36 kDa)
αSMA (42 kDa)
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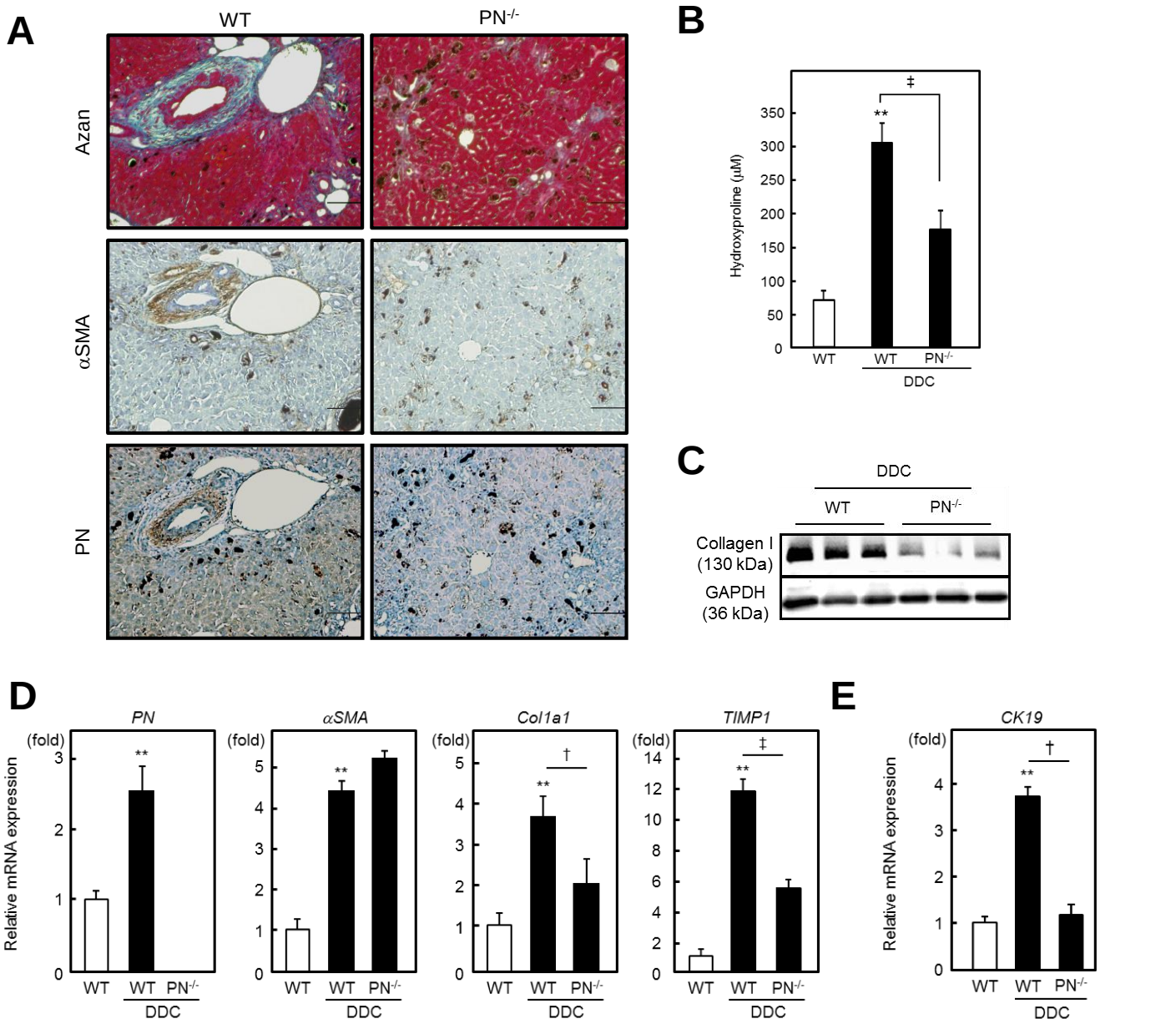
αSMA
Col1a1
TIMP1
(fold)
Relative mRNA expression

Figure 7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TIMP1
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†
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WT
PN-/-
DDC

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