Periostin promotes liver fibrogenesis by activating lysyl oxidase in hepatic stellate cells

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**Running title: Periostin promotes liver fibrogenesis**

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**ABSTRACT**

Liver fibrosis arises from dysregulated wound healing due to persistent inflammatory hepatic injury. Periostin is a non-structural extracellular matrix protein that promotes organ fibrosis in adults. Here, we sought to identify the molecular mechanisms in periostin-mediated hepatic fibrosis. Hepatic fibrosis in periostin**−/−** mice was attenuated as evidenced by significantly reduced collagen fibril density and liver stiffness compared with those in WT controls. A single dose of carbon tetrachloride caused similar acute liver injury in periostin**−/−** and WT littermates, and we did not detect significant differences in transaminases and major fibrosis-related hepatic gene expression between these two genotypes. Activated hepatic stellate cells (HSCs) are the major periostin-producing liver cell type. We found that in primary rat HSCs in vitro, periostin significantly increases the expression levels and activities of lysyl oxidase (LOX) and lysyl oxidase-like (LOXL) isoforms 1-3. Periostin also induced expression of intra- and extracellular collagen type I and fibronectin in HSCs. Interestingly, periostin stimulated phosphorylation of SMAD2/3, which was sustained despite shRNA mediated knockdown of transforming growth factor β (TGFβ) receptor I and II, indicating that periostin periostin-mediated SMAD2/3 phosphorylation is independent of TGFβ receptors. Moreover, periostin induced the phosphorylation of focal adhesion kinase (FAK) and AKT in HSCs. Notably, si-RNA mediated FAK knockdown failed to block periostin-induced SMAD2/3 phosphorylation. These results suggest that periostin promotes enhanced matrix stiffness in chronic liver disease by activating LOX and LOXL, independently of TGFβ receptors. Hence, targeting periostin may be of therapeutic benefit in combating hepatic fibrosis.

Cirrhosis is a late complication of hepatic fibrosis, which results in significant morbidity and mortality worldwide (1-3). Hepatic fibrosis is the consequence of a dysregulated wound-healing process triggered by persistent inflammatory injury to the liver (4). Activated hepatic stellate cells (HSCs), and portal myofibroblasts are the primary cellular players involved in the fibrogenic response to chronic liver injury. In health, quiescent HSCs constitute approximately 8%-14% of the total liver cell population (5,6). Following a fibrogenic stimulus, the quiescent HSC transdifferentiates into a myofibroblast-like cell referred to as the “activated HSC” (4). Activated HSCs secrete a wide array of chemokines and extracellular matrix (ECM) proteins such as fibrillar collagen and fibronectin (4,7). Although key mechanisms of HSC activation have been defined, safe and effective therapeutic targets to
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Periostin is a 90 kDa secretory matricellular protein composed of an N-terminal emilin (EMI) domain, four repeated fasciclin domains, and a C-terminal heparin binding domain with alternatively spliced variants (8,9). Periostin mediates pleiotropic effects during inflammation, tissue injury, and wound healing (8); importantly, it possesses a highly conserved primary sequence structure related to TGF-β-induced protein (βig-h3) (10). In addition to being an ancient wound-healing molecule, periostin is involved in the embryologic development of the periodontal ligament (PDL) (11). Periostin is known to activate canonical signaling pathways during wound healing, tissue repair, and matrix remodeling via transmembrane integrins. However, although integrins serve as receptors for protein containing Arg-Gly-Asp (RGD) sequence, periostin lacks this signature (12). Periostin also has the distinct ability to directly interact with key molecules of fibrotic matrix including fibronectin, type I collagen, elastin, and tenascin via its EMI domain to promote fibrillogenesis (13). This unique property is thought to be responsible for matrix stiffening via collagen cross-linking that ultimately triggers mechanico-signaling to neighboring HSCs. Additionally, ablation of periostin in vivo prevents cardiac, lung, renal and hepatic fibrosis (10,14-16).

Liver stiffness is often associated with excessive accumulation of insoluble and fibrillar collagens in the ECM. Lysyl oxidase (LOX) is a copper-dependent extracellular, matrix-embedded amine oxidase protein that plays a critical role in covalent cross-linking of fibrillar collagens and elastin (17,18). There are a total of five LOX isoforms identified to date: LOX and the LOX-like (LOXL) enzymes 1 to 4, which share a conserved enzymatic domain with divergent N-termini (19,20). LOX, LOXL1, and LOXL2 predominantly promote covalent crosslinking of collagen fibers, which is important for collagen stabilization, as well as matrix integrity and elasticity. Interestingly, LOX expression and activity significantly up-regulates during HSC activation into myofibroblasts (19).

Epithelial-to-mesenchymal transition (EMT) is a multistep dynamic cellular process considered to be involved in the development of lung cancer after periostin exposure (21,22). However, a role for periostin in the process of EMT promotion of liver fibrosis has not been explored.

In this study, we provide several new molecular mechanisms whereby periostin is pivotal in generating the fibrotic response in liver. Deletion of periostin protects from CCl₄-induced liver injury in mice. Periostin induced SMAD2/3, independent of TGF phosphorylation by activation of the integrin αvβ3-PI3kinase pathway.

Results

Periostin⁻/⁻ mice are protected from CCl₄-induced liver fibrosis

In vivo data demonstrated that periostin⁻/⁻ mice are protected from CCl₄-induced liver fibrosis (Fig 1A-F). Since periostin is a non-structural ECM protein known to stimulate type I collagen cross-linking, and promotes matrix stiffness, we examined the stiffness of the mouse livers by atomic force microscopy (AFM). As shown in Fig. 1A, liver stiffness of the CCl₄-treated periostin⁻/⁻ mice was significantly less than the WT controls. The density of collagen fibers in CCl₄-treated periostin⁻/⁻ mice vs WT mice was analyzed by taking transmission electron microscopic (TEM) images of fixed liver tissues as shown in Fig. 1B. The diameter of collagen fibers was measured by Image J (Fig. 1C). By this approach, we demonstrated that collagen fibril density in the periostin⁻/⁻ mice was substantially reduced when compared to the livers of CCl₄-treated WT mice (Fig. 1C).

We examined whether collagen bundles in the fibrotic matrix were qualitatively different in CCl₄-treated periostin⁻/⁻ mice compared to WT controls. For instance the solubility of collagen in the ECM is inversely correlated to the extent of collagen cross-linking. Not surprisingly, the insoluble collagen fraction increased with development of hepatic fibrosis (Fig. 1D). The ratio of insoluble collagens increased from ~5% in untreated groups to ~37% and ~19% in WT and periostin⁻/⁻ mice treated with CCl₄, respectively (P<0.05; Fig. 1D). Additionally, pepsin-soluble collagen decreased from ~90% in untreated controls to ~56% in CCl₄-treated WT mice (Fig. 1D). However, this response to CCl₄ treatment was significantly attenuated in periostin⁻/⁻ mice, which had a reduction in pepsin-soluble collagen to only
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mRNA expression of fibrosis-related genes is reduced in CCl₄-treated periostin⁺/- mice

In addition to histological and biochemical studies, qRT-PCR analysis confirmed significantly attenuated mRNA expression of fibrosis-related genes in CCl₄-treated periostin⁺/- mouse livers, including collagen type I, tissue inhibitor of metalloproteinases 1 (TIMP1) and transforming growth factor-β1 (TGF-β1) (Fig. S2A-D). Both protein and mRNA expression of α-smooth muscle actin (α-SMA), a well-known marker of activated HSCs, and myofibroblasts, was also markedly reduced in CCl₄-treated periostin⁺/- mice compared to WT mice (Fig. S2E-F).

LOX and LOXL1-2 expression is reduced in CCl₄-treated periostin⁺/- mice

Since in vivo data suggested a pivotal role of periostin in collagen fibrillogenesis during the development of liver fibrosis, we next examined whether mRNA expression of LOX and its 4 isoforms LOXL 1-4 changed in the livers of CCl₄-treated periostin⁺/- mice. As shown in Fig. 2A-C, LOX, LOXL1, and LOXL2 mRNA transcripts, respectively, were significantly reduced in the livers of CCl₄-treated periostin⁺/- mice compared to CCl₄-treated WT mice. LOXL3 expression was also induced by CCl₄ treatment, but its expression levels did not differ significantly between WT and periostin⁺/- mice (Fig. 2D). CCl₄-treatment did not change the mRNA expression of LOXL4 in WT and periostin⁺/- mice (Fig. 2E). Protein expression of LOX and LOXL isoforms 1 and 2 was attenuated in periostin⁺/- mice (Fig. 2F). Additionally, we also determined the LOX activity in liver homogenates by fluorometric assay. LOX activity was significantly attenuated in livers from periostin⁺/- mice compared to WT mice (Fig. 2G), both of which were treated with CCl₄ for 6 wk.

Periostin deficiency does not change the liver injury induced by a single CCl₄ treatment

Since periostin⁺/- mice are protected from chronic liver injury, we explored whether the ablation of periostin had any influence on the acute liver response to a single dose of CCl₄ exposure. Our data indicate both periostin⁺/- and WT littermate mice develop centrilobular necrosis which peaked after day 1 following CCl₄ treatment, with recovering by 7 days following CCl₄ exposure (Fig. 3A). We did not observe any significant histological difference between WT and periostin⁺/- mice. Consistent with our histological observations, we also found ALT and AST levels in serum had similar peak values in both periostin⁺/- and WT littersmates at 24 h following the single dose of CCl₄ (Fig. 3B-C). Next, we examined the expression of several genes related to inflammation, fibrosis and matrix remodeling by RT-qPCR. The mRNA levels for TGFβ1, CTGF, TIMP1, Col1α1 and F4/80 were significantly up-regulated after day 1 in both the periostin⁺/- and WT littermate mice (Fig. 3D). Elevated mRNA expression levels persisted through day 3 after injury. Periostin mRNA was significantly up-regulated in WT mice at day 1 and remained up-regulated at day 7. The peak induction in mRNA levels of IL1β, MMP9, MMP13 and IL6 was observed at day 1 in both periostin⁺/- and WT mice, while mRNA of MMP2 was highly up-regulated after day 3 of CCl₄ treatment. We did not observe any significant difference of mRNA expression in either genotype at the indicated time points except TIMP1 and MMP9, which, after day 1, was reduced in periostin⁺/- mice compared to WT littermates (Fig. 3D). Taken together these findings suggest that periostin does not appear to play a significant role in the pathogenesis of acute liver injury unlike chronic injury. Importantly, periostin does not appear to play a major role in the acute inflammatory response.

Periostin expression is up-regulated in CCl₄-induced liver fibrosis and co-localized with α-SMA positive cells in the liver

To investigate periostin expression in major hepatic cell types, and the potential source for periostin, we co-stained live tissue sections of CCl₄-treated WT mice with periostin and markers of major hepatic cell types: albumin (hepatocytes), α-SMA (hepatic stellate cells), CD146 (liver sinusoidal endothelial cells; LSECs) or F4/80 (Kupffer cells). Periostin staining strongly co-localized with α-SMA expressing HSCs, but not with hepatocytes, LSECs or Kupffer cells (Fig. 4 A-D). Quantitative analysis indicated that the percent of overlapping areas (yellow) of periostin

~76% (Fig. 1D). In sum, the matrix stability assessment in the absence of periostin in vivo is consistent with both the AFM and TEM data.
staining (green) with α-SMA positive-staining (Red) was significantly higher than periostin staining which co-localized with F4/80, albumin, or CD146 staining (Fig. 4E).

Next, we isolated and cultured four major cell types (Hepatocyte, HSC, KC and LSEC) from healthy mouse livers. Total mRNA was harvested from each of the cell types after 3 days of primary cell culture. As shown in Fig. 4F, periostin mRNA was expressed at markedly higher levels in HSCs than in hepatocyte, Kupffer cells or LSECs.

**Recombinant periostin up-regulates LOX/LOXL expression in HSCs**

Previous studies have shown periostin can regulate LOX, collagen, and fibronectin expression in fibroblasts (25,26). To dissect the role of periostin in LOX and LOX-related isofrom expression, we isolated primary HSCs from rats and performed immunoblot of lysates from freshly isolated (quiescent), and activated, HSCs as shown in Fig. S3. Culture-activated HSCs highly expressed LOX and its isoforms LOXL1-4 along with periostin, α-SMA, collagen type I and fibronectin (Fig. S3). We treated cultured HSCs with or without full-length human recombinant periostin (100 ng/ml) for 24 h. Supernatant and whole cell lysates were collected for Western blot analysis. In vitro, periostin treatment significantly induced the expression of different LOX isoforms except for LOXL4 (Fig. 5A); and supernatant from cell culture (media) periostin resulted in enhanced secretion of LOXL2 along with fibronectin and collagen type I (Fig. 5B) but not LOXL3 or LOXL4 (data not shown). We confirmed that periostin significantly increased LOX activity in the supernatants of cultured HSCs (Fig. 5C).

**Periostin modulates SMAD2/3 phosphorylation in HSCs**

TGF-β has been reported to induce periostin expression in several cell lines (1,27); however, the relationship between periostin and the TGFβ-SMAD signaling pathway has not been clearly delineated. Furthermore, the prevailing hypothesis is that periostin production is dependent on TGFβ activity. To clarify the association between periostin and the TGFβ-SMAD pathway, we stimulated culture-activated HSCs with various concentrations of periostin for 30 min and performed Western blot analysis followed by immunoblot for p-SMAD2/3 and total SMAD2/3. Our data demonstrated that phosphorylation of SMAD2/3 was significantly increased by periostin in a dose-dependent manner (Fig. 6A).

**Periostin-mediated SMAD2/3 phosphorylation is independent of TGF-β receptors**

To test whether TGF-β receptors (RI and RII) were involved in periostin-mediated SMAD2/3 phosphorylation, we used sh-RNA against both TGF-βRI, and TGF-βRII. Periostin treatment for 30 min significantly induced SMAD2/3 phosphorylation in non-targeted sh-RNA transfected cells (Scrambled), and despite sh-TGF-βRI, or si-TGF-βRII knockdown, periostin-maintained SMAD2/3 phosphorylation (Fig. 6B). We also used a selective inhibitor of TGF-β receptors, LY364947 (10 μm), and performed Western blot analysis of cell lysates derived from culture-activated HSCs exposed to periostin for 30 min. LY364947 failed to block periostin-mediated phosphorylation of SMAD2/3 (Fig. 6C). Collectively, these data suggest periostin-induced serine phosphorylation of SMAD2/3 is independent of TGF-β receptors.

**Periostin induced SMAD2/3 phosphorylation via integrin αvβ3 engagement and PI3kinase signaling**

Previous studies have shown periostin activates integrins which in turn can initiate downstream signaling. We used a blocking antibody against integrin αvβ3 to address whether integrins are mediators of periostin-mediated SMAD2/3 phosphorylation in HSCs. Blocking antibody against integrin αvβ3 significantly attenuated periostin-mediated SMAD2/3 phosphorylation in HSCs (Fig. 6D); and, the phosphoinositide 3-kinase inhibitor (PI3kinase: LY294002) treatment abrogated periostin-induced phosphorylation of SMAD2/3 in HSCs (Fig. 6E).

**Silencing SMAD2/3 attenuates periostin-induced LOX expression in HSCs**

Since periostin-induced the production of LOX, is known to correlate with increased collagen I and fibronectin production, and can independently activate SMAD2/3 in liver myofibroblast, we assessed whether periostin-mediated SMAD2/3 activation was responsible for...
increased LOX expression. By using si-RNA, we knocked down the SMAD2/3 complex in myofibroblasts, and then treated cells with periostin for 24 h. Periostin treatment significantly stimulated the expression of LOX and collagen in scrambled si-RNA transfected HSCs, but periostin-mediated LOX expression was abolished in si-SMAD2/3 transfected HSCs (Fig. 6F). si-SMAD2/3 also attenuated fibronectin and type I collagen culture-activated HSC protein-expression (Fig. 6F).

Integrin αvβ3 is critical to periostin induced LOX expression in HSCs

The above data demonstrate integrin αvβ3 mediates periostin-induced SMAD phosphorylation, and knockdown of SMAD2/3 reduced LOX expression. Therefore, we determined whether integrin αvβ3 was critical to periostin-mediated LOX expression by antibody blockade with anti-αvβ3. As shown in Fig. 7A-B periostin treatment significantly induced LOX expression and activity while anti-integrin αvβ3 blocked both periostin stimulated LOX expression and activity.

si-RNA-mediated knockdown of Focal Adhesion Kinase (FAK) fails to block SMAD phosphorylation in HSCs

Because integrins are known to signal through FAK (28), we next determined whether activation of FAK could promote SMAD2/3 phosphorylation by periostin. For this experiment, we employed siRNA-knockdown of FAK (si-FAK) and performed western blot analysis. As shown in Fig. 8A si-FAK failed to change the status of SMAD2/3 phosphorylation while AKT phosphorylation was attenuated in the presence of periostin (Fig. 8B). Furthermore, blocking antibody against integrin αvβ3 attenuated periostin stimulated phosphorylation of FAK and AKT (Fig. 8B).

Periostin positively regulates epithelial mesenchymal transition (EMT) in primary mouse hepatocytes

Recent data indicate periostin levels are higher in humans with hepatobiliary malignancy (29,30). Since cirrhosis and fibrogenic changes pose risk for tumor development, we explored whether periostin could promote EMT in murine hepatocytes. After isolating mouse hepatocytes, we treated primary cultures with periostin and examined whether periostin influenced the expression of EMT-associated genes. Periostin treatment significantly induced the expression of mesenchymal markers including Col1α1, vimentin, fibronectin and N-Cadherin (Fig. 9A-D). In contrast, the mRNA expression of epithelial markers such as E-Cadherin and HNF4α was effectively suppressed (Fig. 9E-F). We then isolated and cultured hepatocytes from WT and periostin−/− mice and used RT-qPCR to measure EMT related gene expression. Compared to WT mice, hepatocytes of periostin−/− mice expressed significantly higher mRNA levels of epithelial markers and lower mRNA levels of mesenchymal markers (Fig. 9G).

DISCUSSION

In the current study we have demonstrated several molecular mechanisms associated with periostin-mediated liver fibrosis that have not been previously reported. Some of the data presented corroborate recent reports that periostin enhances matrix stiffness and insoluble fibrillar collagen deposition during liver fibrogenesis (16); and our studies lend credence to several reports (16,31,32) that periostin is in contention to be an important pro-fibrogenic mediator. However, here we have shown that periostin may be an independent mediator of SMAD activation which does not appear to be TGFβ dependent. Second, we demonstrated that periostin can activate SMADs via the integrin αvβ3 pathway which is dependent on PI3kinase and is specific to this integrin (Fig. 10). Third, we have shown that periostin plays an important role in the EMT of hepatocytes and that in chronic exposure to CCl4 the bulk of periostin production is derived from HSCs. Together, our new findings clearly merit additional investigation.

Periostin is highly expressed during embryonic development, but expressed at very low levels in adulthood. Recent reports showed that periostin expression was up-regulated in human fibrotic liver tissues compared to healthy controls (16,33). However, periostin−/− mice do not suffer significant developmental abnormalities compared to their WT littermates. Recent studies confirm that periostin deficient mice are protected from fibrotic stimuli in various organs including liver (14,16,27,34). Our current data indicate
fibrotic liver sections of periostin" mice were less stiff than liver sections of WT mice and also have lower levels of collagen I expression. Liver stiffness is a multifaceted phenomenon which makes outside-in-myofibroblast signal transduction (19,20,27) seminal to the molecular orchestration of deposition and maintenance of dense, fibrillar ECM that ultimately leads to disease pathogenesis. As previously reported (35), we have also observed that peristostin can propagate pro-fibrotic signaling via the integrin-PI3kinase signaling axis, specifically via αvβ3 integrins. Earlier studies have shown that collagen cross-linking mediated by the copper-dependent enzyme LOX and its isoforms LOXL1-4 is enhanced by ECM-integrin signaling (36). Mesarwi et al. reported hepatic lox mRNA levels were 6.7 fold higher in patients with fibrosis compared to healthy individuals. Furthermore, they correlated serum lox levels with hepatic mRNA levels (37). It is hypothesized that softer matrices may promote fibrosis resolution and ultimately restore normal matrix density and function by permitting matrix metalloproteinases (MMPs) to ultimately remove dense matrix. Recent studies by Popov et al. (18) and others (38-40) indicate that the LOX inhibitor β-aminopropionitrile (BAPN) may provoke hepatic progenitors to replace the ductular reaction associated with portal fibrosis; hence, there is a strong rationale to investigate additional targets mediating collagen cross-linking in fibrosis injury. These studies are collectively corroborated by recent human studies indicating children who suffer from biliary atresia (BA) have significantly higher circulating levels of periostin (41). Indeed, periostin as a marker for fibrotic injury has long been a surrogate for lung fibrosis (42). Our findings indicate mRNA expression of LOX and its isoforms LOXL1-3 were significantly lower in periostin" mice compared to WT mice after CCl₄ gavage, which makes targeting periostin a compelling strategy for development of antifibrotic therapy.

Taking the current findings together with the other data reported thus far, several important questions need to be addressed. First is whether activated HSCs, and portal myofibroblasts, are the only source of periostin, or do Kupffer cells, or even populations of infiltrating cells play a contributory role in periostin production? Since we note that the typical transaminases associated with chronic CCl₄ exposure is significantly less in periostin" mice, we cannot yet exclude this possibility. Not unexpectedly, we identified HSCs as the major cellular source of persiotin in CCl₄-induced liver fibrosis. The expression of periostin in hepatocytes, LSECs, and Kupffer cells in the CCl₄-treated mice was significantly less than HSCs. Together these observations suggest that HSCs are the major source of hepatic periostin during liver fibrosis, and a definitive source for hepatic periostin production is currently under investigation in our laboratory.

A single dose of CCl₄ treatment resulted in a similar acute response in WT and periostin" mice as demonstrated by serum transaminase levels, histology, and hepatic mRNA levels.

Finally, in the context of these data in periostin" mice, we set out to determine whether periostin possesses additional functions in the pathogenesis of matrix biology beside its known cross-linking function. Our current data support the notion that periostin plays a critical role in enhanced expression and activity of TGF-β1 in HSCs (Fig. S4A-B). We found periostin significantly induced TGF-β1 mRNA expression in HSCs and enhanced its activity in culture supernatants. Also, hepatic TGF-β1 mRNA expression was suppressed in periostin" mice gavaged with CCl₄ compared to CCl₄-treated WT mice. TGF-β1 has been reported to induce periostin expression in several cell lines (1,43,44); however, the biological relationship between TGF-β and periostin is not clear. Given the interrelationship between TGF-β1 and periostin, we sought to determine whether periostin could instigate SMAD activation as a novel ligand for the TGF-β receptor. By both sh-RNA-mediated and pharmacological approaches against both TGF-β receptor isoforms (RI and RII), we found that periostin-induced SMAD2/3 phosphorylation was sustained despite the knockdown the TGF-β receptor as well as in the presence of TGF-β receptors inhibitor. While it may be premature to declare periostin independent of TGF-β without studies performed in animal models, our current data strongly suggest that periostin-induced serine phosphorylation of SMAD2/3 functions independently of TGF-β receptor(s). Periostin also has the unique ability to interact not only with integrins, but has potential to interact with other myofibroblast receptors, e.g. discoidin domain
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In previous studies, it has been demonstrated that periostin contributes to the EMT process in various cancers and fibrosis (21, 46-48). However, the role of EMT in liver fibrosis has become a highly controversial topic in the past several years. In vitro studies demonstrated hepatocytes can undergo the process of EMT and express several mesenchymal markers. Furthermore, Zeisberg et al. reported the first in vivo evidence for hepatocyte EMT in through lineage tracing experiments demonstrating that hepatocytes express EMT markers S100A4 (fibroblast-specific protein 1) (49). Similarly, several other research groups have demonstrated targeting EMT could suppress hepatic fibrogenesis (50-52). However, a recent lineage tracing study identified that mouse hepatocytes fail to undergo EMT during CCl₄-induced liver fibrosis (53). Although this work challenges the existence of hepatocyte EMT in liver fibrosis, lineage tracing techniques have several notable pitfalls. Extensive studies are required to conclude the role of hepatocyte EMT in liver fibrosis.

In this study, we demonstrated that periostin expression appears to be very low in healthy liver, but following a pro-fibrotic stimulus, hepatic periostin expression is markedly increased. The primary source of injury-driven periostin expression at present is the activated HSC, or α-SMA positive myofibroblast. Moreover, periostin−/− mice appear to be protected from CCl₄-induced liver fibrosis, and have reduced liver stiffness. These findings were corroborated with reduced insoluble collagen deposition and diminished LOX/LOXL expression and respective enzyme activity. While there are several novel findings we uncovered here, the results have prompted more questions than we have set out to address. Clearly additional molecular approaches will shed light on whether periostin would be an effective therapeutic target for the treatment of hepatic fibrosis in the future.

Experimental procedures

Animals and CCl₄-induced liver fibrosis in mice

Eight-week old male periostin−/− and wild-type (WT, C57BL/6J) littermate mice were used for animal studies. Periostin−/− mice were purchased from The Jackson Laboratory (Bar Harbor, Maine), Stock no# 009067. The mice used in the experiments were the progeny from backcrossed C57BL/6J mice. Mice were cared for in accordance with protocols approved by the Animal Care and Use Committee of Emory University. Mice were housed in a temperature-controlled environment with a 12:12 h light/dark cycle. Animals were fed ad libitum with Purina Laboratory Standard Chow diet (Ralston Purina, St. Louis, MO) and water. In addition to PCR based genotyping, we also confirmed the absence of periostin in the serum by ELISA (Mouse periostin ELISA kit, RayBiotech, Inc. Norcross, GA).

WT and periostin−/− mice weighing 20-25 g were gavaged with CCl₄ (2 ml/kg) diluted in olive oil (1:1 ratio), or equivalent volume of olive oil mixed with saline thrice weekly for six weeks as described elsewhere (54).

Assessment of liver tissue mechanical properties by atomic force microscopy (AFM)

Liver stiffness was measured by AFM. Liver tissue cryosections of 15 μm thickness were obtained from periostin−/− and WT mice. The AF microscope used in the present study was the MFP-3D (Asylum Research, Santa Barbara, CA) with a Nikon Ti-inverted optical microscope (Nikon, Melville, NY) located at the Georgia Institute of Technology, Atlanta GA. The detail procedure for AFM was described elsewhere (55,56).

Assessment of matrix stability

Matrix stability was analyzed by complete collagen fractionation using a biochemical assay via serial extractions from liver tissues followed by hydroxyproline quantitation. The detail methods of the fibrotic matrix stability assay were described by Popov et al. (18,57).

Primary rat HSCs isolation and treatments

Primary rat HSCs were isolated from male Sprague-Dawley rats by a two-step in situ liver perfusion protocol as described previously (54). Primary rat HSCs were used between passages 2 to 5. Confluent HSCs were serum starved and treated with or without recombinant human periostin (Sino Biological Inc., Wayne, PA) as indicated in figure legends. For inhibitor and
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In a blocking antibody experiment, HSCs were pretreated for 30 minutes followed by stimulation with periostin along with vehicle, control IgG, inhibitor and blocking antibody.

**Lentivirus-mediated TGFβ receptors knockdown**

Plasmids encoding sh-TGF-βRI, sh-TGF-βRII (Cat. #TR709395 and #TR711255), non-targeting shRNA (pRS Vector) and packaging plasmids were purchased from Origene Technologies, Inc. (Rockville, MD). Viral particles were generated by the Lentiviral packing kit (Origene) according to manufacturer’s guidelines. Briefly, HEK293T cells were transfected with premix packaging plasmids and pRS-shRNA lentiviral vector using TurboFectin transfection reagent (Origene). After 48h of transfection, the supernatant containing viral particles were collected and filtered through 0.45 μm PVDF filter. HSCs were transduced with concentrated and purified lentiviral particles (MOI=5) in the presence of polybrene (5 μg/ml). The infected cells were cultured in complete growth medium for 72 h, and stable clones were selected with puromycin dihydrochloride (2 μg/ml). Survived colonies were pooled and expanded in complete cell culture medium containing puromycin (1 μg/ml) for further experiment.

**Hepatocyte isolation**

Hepatocytes were isolated from adult mice using the standard two-step collagenase perfusion method as described previously (58). Briefly, under anesthesia, liver was perfused in situ via the portal vein for 5 minutes with calcium-magnesium-free HEPES buffer. Liver was digested with magnesium-free buffer containing type IV collagenase (30 mg/ml) and calcium chloride (10 mM) for 6 minutes. Digested liver was teared with the help of sterile forceps. Cells were filter through 70 μm nylon cell strainers (Thermo Fisher, Waltham, MA). Cells were centrifuge at 50 g for 2 minutes and supernatant was discarded. Pellet was resuspended in 20 ml of 40% percoll followed by centrifugation at 200 g for 5 minutes. After centrifugation, bottom parts (hepatocytes) were collected. Cells were wash three times with 10 ml of DMEM containing 5% FBS by centrifugation at 50 g for 2 minutes.

**Statistical analysis**

All data are expressed as the mean ± standard error (SE) from 3-4 separate experiments. The differences between groups were analyzed using a two-tailed Student t test when only two groups were analyzed or analysis of variance (ANOVA) when there were more than two groups were analyzed. The statistical analyses were conducted using the Excel (Microsoft office 2015) Graph-Pad Prism software version 5.04 (GraphPad® Software, San Diego CA). A P value <0.05 was considered as statistically significant differences.
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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: PK: Study design, data collection, performed all the experiments, statistical analysis and interpretation of data and wrote the manuscript. TS: maintained mouse colony and CCl4 gavage. RR: critically revised the manuscript for important intellectual content. DMC: critically revised the manuscript for important intellectual content. HB: acquired AFM data Sulchek: Provided AFM data. YL: Provided assistance in Western blot analyses. FAA: Study concept and design, outline and revised the manuscript.
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Figure 1: Periostin−/− mice are protected from chronic CCl₄-induced liver fibrosis. (A) AFM images show CCl₄-treated periostin−/− mouse liver sections are less stiff than CCl₄-treated WT mice. (B) TEM images of livers from WT and periostin−/− mice treated with CCl₄ for 6 weeks (scale bar: 0.2 µm). (C) The diameter of the collagen fibers (n=3). (D) Liver fibrotic matrix stability assay demonstrating significantly higher cross-linked and insoluble collagens in CCl₄-treated WT mice compared to periostin−/− mice. Results are expressed as mean value ± SEM. n= 8/group; *P< 0.05 compared to control group; #P< 0.05 compared to WT mice treated with CCl₄ group to periostin−/− mice treated with CCl₄.
Figure 2: mRNA expression of fibrosis and LOX(s) related genes in liver lysates were reduced in CCl₄-treated periostin⁻/⁻ mice compared to WT mice. The relative mRNA expression was quantified by qRT-PCR in WT (clear bars) and periostin⁻/⁻ (black bars) after 6 weeks of CCl₄-treatment. (A-E) mRNA expression of LOX and LOXL associated isoforms. The relative mRNA data represent the expression of the respective gene relative to the expression of 18s used as an internal control. (F) Representative Western blots images of LOX and LOX1-2 expression. (G) LOX activity measured in liver lysates. Results are expressed as mean value ± SEM. n=8/group; *P< 0.05 compared to control group; #P< 0.05 compared to WT mice treated with CCl₄ group to periostin⁻/⁻ mice treated with CCl₄.
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Figure 3: Acute liver injury modulates similar liver injury and gene expression in periostin-/- and WT mice. Periostin and WT mice were treated with CCl₄ and sacrificed after 1 day, 3 days and 7 days. (A) Representative images of hematoxylin and eosin staining of liver sections (magnification X10). (B-C) Serum ALT and AST activity levels are shown as international units per liter. (D) The hepatic mRNA expression of several genes related to fibrosis, inflammation, and matrix remodeling. Results are presented as mean value ± SEM of groups from 4-5 mice in fold change compared to control WT mice. No significant difference was observed between the periostin-/- and WT mice for gene expression at any time, except for TIMP1 and MMP9 after day 1. *P < 0.05 compared to control group; #P < 0.05 compared to WT mice treated with CCl₄ group to periostin-/- mice treated with CCl₄.
**Figure 4:** Activated HSCs are the major source of periostin in CCl₄-induced liver fibrosis. (A-D) Immunofluorescent staining for co-localization of periostin with α-SMA (marker for activated HSCs), albumin (marker for hepatocytes), F4/80 (marker for Kupffer cells), or CD146 (marker for liver sinusoidal endothelial cells; LSECs) in liver sections obtained from control and CCl₄-treated WT mice. Scale bar 50 µm. (E) The percentage of the merged area (yellow) compared to periostin stained-liver (green) in CCl₄-treated mice. Data are expressed as mean value ±SEM. *P < 0.05 compared to α-SMA positive staining. (F) Four different liver cell types were isolated from healthy mouse livers. Cells were cultured in appropriate medium for 5 days and total mRNA was extracted from different cell types. Periostin mRNA expression in primary mouse hepatocytes, HSCs, Kupffer cells and LSECs was determined by RT-PCR.
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Figure 5: Periostin treatment up-regulates expression of LOX and LOXL1-3 in primary rat HSCs. (A) Periostin treatment for 24 h significantly induced LOX, LOXL1-3, collagen and fibronectin expression in HSCs. (B) Periostin treatment for 24 h significantly increased extracellular expression of LOXL2, collagen, and fibronectin in culture supernatant of HSCs. (C) LOX activity was measured in supernatants of HSCs in culture. Densitometric analysis was performed for quantification of data expressed as relative arbitrary units. These data are representative of three independent experiments. *P < 0.05 compared to control.
Figure 6: Periostin induced phosphorylation of SMAD2/3 which was sustained in the face of molecular or chemical inhibition of both TGF-βRI and TGF-βRII function and periostin induced SMAD2/3 phosphorylation is mapped to the integrin αβ3/PI3kinase axis. (A) Periostin treatment for 30 min increases phosphorylation of SMAD2/3 in a dose-dependent manner. (B) sh-TGFβ-R1 and -R1I fails to block periostin induced SMAD2/3 phosphorylation. (C) TGF-β receptor inhibitor (LY364947) also fails to block periostin-induced SMAD2/3 phosphorylation in HSCs. (D) Blocking antibodies against integrin αβ3 inhibits periostin-induced SMAD2/3 phosphorylation in HSCs. (E) PI3kinase inhibitor LY294002 (20 μM) prevents periostin-induced SMAD2/3 phosphorylation in HSCs. (F) si-SMAD2/3 blocks the periostin-induced LOX, collagen and fibronectin expression in HSCs. Densitometric analysis was performed for quantification of data expressed as relative arbitrary units. These data are representative of three independent experiments. *P < 0.05 compared to control and #P < 0.05 compared to periostin-treated HSCs.
**Figure 7**: Blocking antibody anti-integrin αvβ3 attenuates periostin-induced LOX (s) expression and LOX activity. (A) Primary HSCs were treated with or without periostin and IgG or blocking antibody integrin αvβ3 for 24 h and performed Western blot analysis. (B) LOX activity was measured in supernatants from activated HSC cultures. Densitometric analysis was performed for quantification of data expressed as relative arbitrary units. These data are representative of three independent experiments. *P < 0.05 compared to control and #P < 0.05 compared to periostin-treated HSCs.
Figure 8: si-FAK fails to block periostin-induced SMAD2/3 phosphorylation in HSCs. (A) Representative western blot analysis of SMAD2/3 and AKT phosphorylation in the presence of periostin after knockdown of FAK by siRNA. (B) Western blot analysis of serum-starved HSCs was pre-treated with IgG or anti-integrin αvβ3 for 30 minutes followed by periostin treatment along with IgG or anti-integrin for 30 minutes. Blocking antibody integrin αvβ3 significantly inhibits periostin-mediated phosphorylation of FAK and AKT. *P < 0.05 compared to control and #P < 0.05 compared to periostin-treated HSCs.
**Figure 9:** Periostin involved in epithelial-mesenchymal transition (EMT) related genes expression in mouse primary hepatocytes. (A-D) Periostin treatment of primary mouse hepatocytes for 24 h induced mesenchymal marker assessed by RT-qPCR. (E-F) Periostin treatment reduced mRNA expression of epithelial marker in hepatocytes. (G) Relative mRNA expression of epithelial and mesenchymal marker in culture hepatocytes (72 h) compared to WT hepatocytes. Experiment were performed twice and are shown as mean value ± SEM. *P< 0.05 compared to untreated hepatocytes (A-F) and compared to WT hepatocyte (G).
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Figure 10: The profibrogenic effects of recombinant periostin mediated by integrin-αvβ3-PI3kinase axis. Periostin induced collagen expression in HSCs via integrin αvβ3 and activates PI3kinase followed by SMAD2/3 phosphorylation. SMAD2/3 phosphorylation leads LOX and LOXL1-3 activation and collagen production and crosslinking.
Periostin promotes liver fibrogenesis by activating lysyl oxidase in hepatic stellate cells
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