Role of Smad3 in Platelet-Derived Growth Factor-C induced liver fibrosis

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Running Header: Loss of Smad3 blocks fibrosis

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Disclosures: none

Keywords: PDGF, TGFβ, Smad, liver fibrosis, hepatic stellate cell

Abbreviations:
Platelet-derived growth factor, PDGF; transforming growth factor beta, TGFβ; TGFβ receptor 1, TGFβR1; Smad, mothers against decapentaplegic homologue (MADH); PDGF receptor alpha or beta, PDGFRα, PDGFRβ; transgenic, Tg; knock-out, KO; wild type, WT; extracellular matrix, ECM; intraperitoneal, IP; bromodeoxyuridine, BrdU; non-parenchymal cell, NPC; hepatocellular carcinoma, HCC; hepatic stellate cells, HSCs; smooth muscle alpha actin, aSMA; connective tissue growth factor, CTGF; cellular retinol binding protein 1, CRBP1; matrix metalloproteinases, MMPs; epidermal growth factor, EGF.
Abstract

Chronic liver injury leads to fibrosis and cirrhosis. Cirrhosis, the end stage of chronic liver disease, is a leading cause of death worldwide, and increases the risk of developing hepatocellular carcinoma. Currently there is a lack of effective anti-fibrotic therapies to treat fibrosis and cirrhosis. Development of anti-fibrotic therapies requires an in-depth understanding of the cellular and molecular mechanisms involved in inflammation and fibrosis after hepatic injury. Two growth factor signaling pathways that regulate liver fibrosis are transforming growth factor beta (TGFβ) and platelet-derived growth factor (PDGF). However, their specific contributions to fibrogenesis are not well understood. Using a genetic model of liver fibrosis, we investigated whether the canonical TGFβ signaling pathway was necessary for fibrogenesis. PDGF-C transgenic (PDGF-C Tg) mice were intercrossed with mice that lack Smad3, and molecular and histological fibrosis was analyzed. PDGF-C Tg mice that also lacked Smad3 had less fibrosis and improved liver lobule architecture. Loss of Smad3 also reduced expression of collagen genes, which were induced by PDGF-C, but not the expression of genes frequently associated with hepatic stellate cells (HSCs) activation. In vitro HSCs isolated from Smad3 null mice proliferated more slowly than cells from wild type mice. Taken together these findings indicate that PDGF-C activates TGFβ/Smad3 signaling pathways to regulate HSC proliferation, collagen production and ultimately fibrosis. In summary, these results suggest that inhibition of both PDGF and TGFβ signaling pathways may be required to effectively attenuate fibrogenesis in patients with chronic liver disease.
**Introduction**

Chronic liver injury results in liver fibrosis, which may progress to cirrhosis(31, 42, 45), increasing the risk of developing hepatocellular carcinoma (HCC)(52). Liver fibrosis, a maladaptive form of wound healing, results from the interplay of various intra- and extra-hepatic cells activated by a wide variety of cytokine and growth factor mediators(15, 23). Hepatic stellate cells (HSCs) and portal fibroblasts are recognized as the primary cellular mediators of fibrosis as they produce collagen(15, 29, 42, 57). Understanding the molecular pathways involved in coordinated activation and inactivation of HCSs and/or myofibroblasts in fibrogenesis is critical to the development of anti-fibrotic therapies.

A plethora of cytokines, chemokines, growth factors and extracellular factors are involved in HSC activation, proliferation and migration(22, 46, 59). Two families of growth factors appear to be critical regulators of HSCs; transforming growth factor beta (TGFβ) ligands, and platelet derived growth factor (PDGF) ligands(46). The PDGF ligand family, PDGF-A, B, C, and D, transmit their extracellular signals via tyrosine kinase receptors, PDGF receptor α (PDGFRα) and β (PDGFRβ)(1, 5). Stimulation of PDGF receptors induces HSCs proliferation in vitro(44, 60), and overexpression of PDGF-A, -B, or -C in the liver(7, 9, 53) results in fibrosis. Hepatic overexpression of PDGF-C results in progressive liver disease, development of tumors, and decreased life span(7), phenotypes not seen when PDGF-A or -B are overexpressed in the liver(9, 53). It is not yet known how the overexpression of these different PDGF ligands results in different liver pathology. Thieringer and Czochra have suggested that the different outcomes may reside with differences in PDGF ligands to stimulate transforming growth factor beta (TGFβ) ligands(7, 9, 53).

TGFβ is a multifunctional cytokine that regulates cell survival, differentiation, migration and synthesis of extracellular matrix (ECM) components (12, 25, 58). Elevated levels of TGFβ are seen in organ fibrosis and, Tgfb overexpression results in liver fibrosis(50) (27). TGFβ stimulates type I collagen production, the hallmark of liver fibrosis, as well as regulating fibrinolysis factors, such as matrix metalloproteinases (MMPs) and their inhibitors(12, 25, 58). TGFβ binds to cell surface receptors to initiate a number of intracellular signal transduction pathways, including activation of Smad proteins, which are transcription factors(12, 25, 58). TGFβ ligand binding stimulates receptor phosphorylation of Smad2 and 3, allowing binding with Smad4 forming complexes that translocate to the nucleus to activate gene expression. Among the genes induced by Smad2/Smad3:Smad4 complexes is the inhibitory Smad, Smad7. Smad7 is regulated by Smad2/3 transcriptional activity and disrupts receptor activation of Smad2/3, which inhibits TGFβ signaling in a negative feedback loop (34, 38, 39).

We have previously reported that hepatic overexpression of PDGF-C results in progressive liver fibrosis, which increases in severity as the mice age, and eventually the development of HCCs(7). Shortly after birth, extensive HSC activation and proliferation is
apparent, accompanied by elevated hepatic Tgfβ1(7, 8). Correlation between collagen deposition, HSC activation and elevated levels of Tgfβ1 suggests that TGFβ may be critical regulator in this mouse model of fibrosis. In other hepatic transgenic PDGF mouse strains, increased expression of TGFβ was reported for PDGF-A(53), but not PDGF-B(9). Thus, it is plausible that differences in the amount of TGFβ produced in each of these models(7, 9, 53) may account for the observed differences in the severity of chronic liver disease in these models.

In this study we sought to investigate whether TGFβ regulates PDGF-C-induced liver fibrogenesis by interfering with TGFβ signaling pathways, by deleting the Smad3 allele. To test this hypothesis, we generated PDGF-C Tg mice that also lacked Smad3 and found that PDGF-C;Smad3 KO mice had less fibrosis compared to PDGF-C Tg mice. Gene expression studies revealed that collagen gene expression was Smad3-dependent, while genes associated with HSCs activation were not significantly decreased in PDGF-C;Smad3 KO mice. Isolated Smad3 null HSCs grew more slowly than wild type HSCs. In vitro, PDGF ligands stimulated TGFβ protein production and release. In summary, these data indicate that Smad3 is an important mediator of PDGF-C-induced fibrosis, and delineated the contributions of TGFβ/Smad3 pathways in this model. PDGF-C regulates both Smad3-dependent and independent signaling pathways. Thus, combination therapy that targets both TGFβ- and PDGF signaling pathways may provide the most effective long-term therapy for chronic liver disease and prevention of HCC.

Materials and Methods

Generation of PDGF-C Tg;Smad3-deficient mice

PDGF-C transgenic (PTg) mice(7) and Smad3 deleted mice with targeted disruption of exon 8 in the Smad3 gene (Smad3KOex8/ex8) have been previously described(62). Prior to initiating the experiments described in this study, Smad3KOex8/ex8 were cleared of helicobacter infection by neonatal re-derivation(54), verified by PCR on DNA extracted from fecal matter(35). Additionally Smad3KOex8/ex8 mice, originally on a mixed 129 background, were backcrossed onto a C57BL6.Jax background for six or more generations. To generate experimental mice, wild type (WT, non-transgenic), heterozygous Smad3 (i.e. PDGF-CWT/WT; Smad3ex8/WT) females were bred with PDGF-C Tg, heterozygous Smad3 gene (i.e. PDGF-CTg/WT; Smad3ex8/WT) male mice. Offspring were genotyped by PCR on DNA extracted from tail tips. Helicobacter-free male and female mice were sacrificed by CO₂ inhalation for evaluation of liver histology, liver cell proliferation by BrdU incorporation(7), gene or protein expression, or HSC isolation between the ages of two and six months. As previously described, Smad3 null mice were smaller than hemizygous or WT littermates(62). All animal studies were carried out under approved Institutional Animal Care and Use Committee protocols from the University of Washington,
which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. For the remainder of the manuscript, mice will be referred to as WT, KO (Smad3 KO ex8/ex8), PTg (PDGF-C Tg), and PTg/KO (PDGF-C Tg; Smad3 KO ex8/ex8).

Liver histology and immunohistochemistry
Mouse livers were fixed in 10% neutral-buffered formalin or Methyl Carnoy’s solution (60% methanol, 30% chloroform and 10% acetic acid, V:V:V) overnight, processed to paraffin blocks, sectioned, and stained with hematoxylin/eosin, Masson’s Trichrome or picrosirus red using standard techniques. Staining was performed as previously described using antibodies specific for mouse anti-BrdU (7) (Dako, Carpinteria, CA) and rabbit anti-phospho-Smad2/3 (Zymed, now Life Technologies Inc.). The Zymed antibody detects both Smad2 and Smad3. Detection of primary antibodies was done using the appropriate biotinylated antibody (Vectastain Burlingame, CA) and visualized with a peroxidase diaminobenzidine kit (Ventana, Tucson, AZ). The mouse on mouse kit (Vectastain) was used to detect BrdU labeling of both non-parenchymal cells (NPCs) and hepatocytes as a measure of cell proliferation (7, 8). Data are represented as the number of BrdU positive hepatocyte nuclei or NPCs observed in thirty 40X fields (i.e. 1.3 mm²; approximately 3,000 hepatocytes). Morphometric analysis was performed as described previously (8, 48).

RNA isolation and real-time RT-PCR analysis
RNA was isolated from whole liver snap frozen at the time of necropsy using TRIzol (Invitrogen, now Life Technologies, Carlsbad, CA) according to the manufacturer’s recommendation. For cDNA synthesis, 2 µg RNA was reverse transcribed using the Retroscript kit (Life Technologies, Carlsbad, CA), and real-time RT-PCR (qPCR) was performed using FAM-labeled primers (Applied Biosystem, Forster City, CA) as described or primers used with Sybr Green (primer sequences are available on request). Gene expression data are normalization to Gapdh mRNA or 18S rRNA levels using the ∆∆Ct method as previously described (61).

Protein extraction and immunoblotting
Whole liver lysates were prepared using a Triton-X 100 lysis buffer with protease inhibitors and quantified using the Bradford method with BSA (8). SDS-PAGE analysis of proteins levels were determined using immunoblotting and the following antibodies: rabbit anti-Smad3 (Zymed, Carlsbad, CA), rabbit anti-Smad2 (Zymed, South San Francisco, CA), mouse anti-Smad4 (Santa Cruz, Santa Cruz, CA), mouse anti-human αSMA (DAKO, Carpinteria, CA), and rabbit anti-GAPDH conjugated to HRP (GeneScript, Piscataway, NJ). Epitope-primary antibody complexes were detected with species-specific secondary antibodies conjugated to HRP followed by ECL (Thermo Fisher Scientific Pierce, Illinois).
Primary HSC isolation and culture conditions
HSCs were isolated from WT and KO mice (i.e. Smad3<sup>ex8/ex8</sup>), using pronase/collagenase (36, 56) perfusion and density centrifugation methods using Nycodez. Isolated HSCs were cultured on uncoated tissue culture dishes (1-4 x 10<sup>4</sup> cells/cm<sup>2</sup>) in DMEM supplemented with 10% FBS and penicillin in 95% air 5% CO<sub>2</sub> humidified atmosphere at 37°C. After seven days, cultures were placed in serum-free media and incubated with serum-free media or with PDGF-CC in serum-free media for 24 hrs. Typically isolated HSCs were combined from two to three mice. Stocks solutions of TGFβ (R&D Systems) were prepared according to the manufacturer’s directions.

Cell proliferation in primary HSCs cells.
[^3H]Thymidine incorporation or cell counting were used to measure cell proliferation as previously described (3, 7). Primary HSCs were isolated from 6 to 7 week old WT mice or Smad3 KO mice and maintained as described in the previous section. In some experiments HSC cultures were serum starved overnight prior to ligand treatment and subsequent addition of[^3H]Thymidine.

Detection of TGFβ1 in HSC media.
Rat CFSC-2G stellate cell cultures were maintained as described by Greenwel and co-workers (18). CFSC-2G cells were serum-starved overnight with BSA, treated with the indicated doses of PDGF-CC and –DD (a gift from ZymoGenetics(17)) -AA, -AB or –BB (R&D Systems), and cell culture media was collected 24 to 72 h later. TGFβ protein levels were determined by ELISA (R&D Systems Quantikine Human Immunoassay cat# DB100B) after media was treated with HCl to convert latent TGFβ to the active form. This assay recognizes human porcine, mouse, rat and canine TGFβ1, but not other TGFβ isoforms.

Statistical analysis
All results are shown as mean ± standard error of the mean (SE). Data were analyzed using non-parametric analyses, including Mann-Whitney test, or Kruskal-Wallis with multiple comparisons with Dunn’s posttest. P<0.05, (*) was considered as statistically significant. Statistical analyses were performed with SPSS version 12.0 software for Windows (SPSS Inc., Chicago, IL, USA) or Prism (GraphPad Software, CA, USA).

Results
Elevated TGFβ/Smad signaling in fibrotic liver tissue from PDGF-C Tg mice. PTg mice develop pericellular and perivenular hepatic fibrosis that progresses to bridging fibrosis as
the mice age(7, 8). As TGFβ is a potent pro-fibrotic molecule in many different tissues(13, 16), we hypothesized that TGFβ signaling pathways contributed to the development and progression of fibrosis observed in this model. Previously, we observed that Tgfβ1 mRNA was elevated in PDGF-C PTg mice compared to WT littermates(7). Additionally, cDNA microarray analysis revealed that genes in the TGFβ signaling pathway, including several Smads, were significantly up-regulated in liver tissue from PDGF-C PTg relative to WT mice(61) (GEO number GSE38199). To provide further evidence of TGFβ signaling, we performed real-time PCR, immunoblot and immunohistochemical analyses on liver tissue from eight to nine week old WT and PTg mice (Fig.1). We observed elevated levels of TGFβ1 receptor 1 (Tgfβr1) mRNA (Fig.1,A1), Smad3 (Fig. 1A.2), but not Smad2 (Fig. 1, A.3) in PDGF-C PTg compared to WT mice. In addition, expression of Smad7, a transcriptional target of Smad2/3, was also induced (Fig.ure 1, A.4); demonstrating that components of the TGFβ signaling pathway were present in livers of PDGF-CP Tg mice.

Immunoblot analysis of Smad2 and Smad3 showed increased protein levels of Smad3, but not Smad2 in PTg mice (Fig. 1B). In addition phosphorylated Smad2/3 positive staining was detected by IHC in PTg mice (Fig. 1C2 and 1C.4), whereas little nuclear staining was appreciated in liver tissue from WT mice (Fig. 1.C1 and 1C3). Non-parenchymal cells (NPCs) also appeared to contain phosphorylated Smad2/3, but there was insufficient resolution to determine whether Smad2/3 had translocated into the nucleus. Together, these results suggest that TGFβ signaling pathways were active in livers from PTg mice, and that Smad3 may play a role in mediating TGFβ signaling pathways in this mouse model.

Based on these in vivo observations, we wondered whether PDGF-C could directly stimulate TGFβ production in vitro. It is well known that TGFβ is a potent stimulator of PDGF ligand production in a variety of cell types (5, 41, 46), but less is known about the ability of PDGF-C to stimulate TGFβ production. Rat CFSC-2G stellate cells(18) were exposed to PDGF-CC to determine whether this ligand stimulates TGFβ production in an established stellate cell line PDGF-CC treatment resulted in TGFβ1 protein release into the media with PDGF-AB, -BB and –CC ligands being more efficacious than –AA or –DD (data not shown). These results demonstrate that PDGF ligands are capable of stimulating TGFβ release in vitro in an activated myofibroblast-like cell line.

**Deletion of Smad3 in PDGF-C Tg mice reverses phenotypic changes induced by PDGF-C.**

If PDGF-C-induced liver fibrosis is dependent on TGFβ production and TGFβ regulates collagen production, then disruption of TGFβ signaling pathways should attenuate fibrosis in PTg mice. To test this hypothesis, we intercrossed PTg mice with mice that lack Smad3(62), a transcription factor that is part of TGFβ canonical signaling. Based on our breeding scheme, six different genotypes were possible, and the following four genotypes were analyzed in detail;
wild type (WT, PDGF-C\textsuperscript{W}/Smad3\textsuperscript{W}), Smad3 KO (KO, PDGF-C\textsuperscript{W}/Smad3\textsuperscript{K}), PDGF-C Tg (PTg, PDGF-C \textsuperscript{Tg}/Smad3\textsuperscript{W}), and PDGF-C Tg; Smad3KO (PTg/KO, PDGF-C\textsuperscript{Tg}/Smad3\textsuperscript{K}). Over the course of these studies, we observed fewer than expected Smad3KO mice. Mendelian analysis of pups at weaning indicated that mice that lacked the Smad3 allele were born less frequently than expected (Table 1). The higher mortality of Smad3 null weanlings appears to be independent of PDGF-C transgene as PTg/ KO mice were born at nearly the same frequency as KO mice.

WT, KO, PTg and PTg; KO mice were sacrificed and analyzed to determine the effects of Smad3 deletion on PDGF-C-induced changes in the liver (Fig. 2). We first examined whether deletion of Smad3 resulted in loss of Smad3 protein. Immunoblot analysis of liver lysates from all four genotypes showed that PTg mice had the highest levels of Smad3 protein, approximately twice the amount seen in WT mice (Fig. 2A). Smad3 protein was not detectable in lysates from KO or PTg/KO mice. Smad2 protein levels did not change with overexpression of PDGF-C or deletion of Smad3 (Fig. 2A), and SMAD4 protein levels were also unchanged (data not shown). These results indicate that PTg/KO mice are deficient for Smad3, but have similar levels of Smad2 and Smad4 as WT, KO and PTg mice.

PTg mice overexpress the full-length human PDGF-C transgene with an intact CUB domain. For PDGF-C to activate PDGF receptors, the CUB domain must be cleaved from the growth factor domain by extracellular proteases(48, 61). To evaluate circulating levels of human PDGF-CC by ELISA, plasma was collected from mice representing all four genotypes. Only PTg mice had circulating levels of active ligand, and no significant differences were seen between PTg and PTg/KO mice (Fig. 2B). Thus, changes in transgene expression do not appear to account for differences in observed liver phenotypes.

PTg mice have greater liver weight to body weight ratios than WT littermates(17), and systemic Smad3 deletion resulted in a statistically significant decrease in liver weight/body weight in PTg mice (Fig. 2C). PDGF-C overexpression also increases the spleen weight relative to body weight, a phenotype that was also partially reversed by deletion of Smad3 (Fig. 2D). Liver cell proliferation is enhanced in PTg mice(7, 8); deletion of Smad3 decreased both hepatocyte (Fig. 2E) and NPC proliferation (Fig. 2F) as measured by BrdU incorporation. No differences in liver cell proliferation were observed between WT and KO mice. These results indicate that TGFβ/Smad3-dependent pathways are in part responsible for prominent cellular changes seen in PTg mice. Moreover, these data indicate that Smad3 signaling is ‘downstream’ of PDGF-C signaling in this model, as loss of Smad3 partially reverses PDGF-C-induced changes in the liver.

Smad3 null HSCs proliferate slowly.
In this study we have shown that NPC proliferation in vivo was significantly decreased in
PTg/KO mice compared with PTg mice (Fig. 2F). We thus wondered whether TGFβ effects could be observed in isolated primary HSCs from KO mice would be differentially affected by TGFβ (Fig. 3). In vitro, TGFβ has different effects on cell growth depending on a variety of factors, including the embryologic origin of the cell and its degree of activation or transformation. Mesenchymal cells such as myofibroblasts proliferate or trans-differentiate in response to TGFβ(5, 46, 58).

To evaluate the effect of Smad3 gene deletion on HSC proliferation, HSCs were isolated from both WT and KO mice, and cell proliferation was assessed. Primary HSCs isolated from KO mice grew more slowly on plastic dishes than WT HSCs (Fig. 3A). WT cells doubled in approximately two days, while Smad3 KO HSCs took four days nearly twice as long to double in number. Nonetheless Smad3 null HSCs were able to proliferate, albeit slowly. To evaluate the effect of Smad3 gene deletion on PDGF-CC-stimulated HSC proliferation, we next plated cultured primary HSCs from WT and Smad3 KO mice on plastic for seven days, which allowed them to become activated and myofibroblast-like. After removing the serum, the cultures were exposed to PDGF-CC and cell proliferation was measured. PDGF-CC-induced HSC proliferation was significantly attenuated in Smad3 null HSCs (Fig.3B). Thus Smad3 null HSCs grow more slowly than WT HSCs in the presence of either serum or PDGF-CC in culture. These in vitro results mirror the decreased NPC proliferation observed in vivo in livers of PTg/KO mice (Fig. 2F).

Deletion of Smad3 ameliorates PDGF-C induced liver fibrosis.
To investigate the role of Smad3 on PDGF-C-induced liver fibrosis, collagen deposition was examined in fixed liver tissues using Masson’s trichrome and picrosirius red staining (Fig. 4). Architectural derangement of liver and deposition of fibrous filaments was apparent in PTg mouse specimens, but was diminished in PTg/KO mice (Fig. 4C and 4D). Collagen staining was attenuated in 3-month-old PDGF-C Tg;Smad3 KO mice compared to PDGF-C Tg mice as detected by trichrome (Fig. 4A and 4C) or picrosirus red (Fig. 4B and 4D) staining. Liver specimens from WT and Smad3 KO mice had little collagen deposition, which was less than that seen in PDGF-C Tg;Smad3 KO mice. Differences in collagen deposition were not apparent between WT and Smad3 KO strains (data not shown).

To quantify the differences in collagen deposition, we performed morphometric analysis on picrosirius red stained liver tissue from WT, KO, PTg and PTg/KO mice (Fig. 5). Liver fibrosis was significantly decreased in PTg/KO mice compared to PDGF-C PTg mice. Loss of Smad3 in PTg mice did not fully reverse the collagen deposition to the levels seen in uninjured WT and KO mice, however. Together these results indicate that TGFβ/Smad3 signaling pathways partially regulate drive PDGF-C-induced fibrogenesis.

Smad3 regulates hepatic gene expression in PDGF-C Tg mice.
Smad3, a critical component of the canonical TGFβ signaling pathway, transcriptionally regulates expression of genes that contributes to fibrosis. To assess the consequences of Smad3 deletion on PDGF-C-induced changes in gene expression, we performed real-time RT PCR analyses using whole liver RNA from WT, KO, PTg and PTg/KO mice (Fig. 6). PDGF-C overexpression induces collagen α1(I) (Col1a1), collagen α2(I) (Col2a1), and collagen α1(IV) (Col4a1) expression in the livers of PTg mice, but this induction was blocked in PTg/KO mice (Fig. 6A). Increased smooth muscle α-actin (αSMA, Acta2) is a hallmark of liver fibrosis and is associated with HSC activation. PTg/KO mice have less Acta2 mRNA than PTg mice. Connective tissue growth factor (CTGF, Ctgf) is regulated by TGFβ and is implicated in the regulation of a number of collagen genes (40, 47). PDGF-C overexpression up-regulates Ctgf expression, which is attenuated in PTg/KO mice.

**PDGF-C Tg mice induce hepatic genes that are Smad3-independent.**

We have previously reported the overexpression of PDGF-C results in the induction of number genes, including Tgfβ1 and the PDGF receptors, Pdgfra and Pdgfrb (7, 61). In these experiments, we observed a similar induction of these genes in PTg mice, but their expression was not significantly changed when Smad3 was deleted in PTg/KO mice (Fig. 6B). These results suggest that Smad3-independent- or additional signaling pathways regulate cellular retinol binding protein 1 (Crbp1), Tgfβ1, Pdgfra and Pdgfrb gene expression in the PTg liver. Since these genes are often associated with HSC activation and/or proliferation (25, 26, 43, 59), our results suggest that HSC activation may be dependent on PDGF-C-mediated pathways to a greater extent than those mediated by Smad3.

**Discussion**

TGFβ and PDGF signaling pathways are important regulators of liver fibrosis. However, how these signaling pathways collaborate in liver fibrogenesis is not well understood. The major finding of this study is that PDGF-C-induced hepatic fibrosis is dependent on TGFβ signaling that is regulated by Smad3. Overexpression of PDGF-C results in activation of HSCs, and fibrosis, which increases with severity as Tg mice age. As Tgfβ1 levels are elevated in PDGF-C Tg mice compared to WT mice, we suspected that part of the fibrotic potency of PDGF-C was due to activation of TGFβ signaling pathways. Using a genetic approach, we showed that PTg mice that lack Smad3 have decreased liver and spleen mass, and less collagen deposition compared to PTg mice with intact Smad3. Using in vitro approaches we show that PDGF ligands have the capability to stimulate TGFβ production. These data suggest the following sequence of events in PDGF-C-induced fibrosis. PDGF-C induces Tgfβ expression, which drives the expression and deposition of collagen, resulting in fibrosis. In addition to TGFβ’s effects on HSCs, PDGF-C also directly stimulates the proliferation and activation of HSCs.
Our findings are consistent with previous studies in liver fibrosis indicating that Smad3 is a key regulator of collagen deposition. Schnabl and co-workers(51) showed that Col1a1 expression was decreased, while αSMA expression was not after acute carbon tetrachloride (CCl4) injury in Smad3KO×ex8 mice. In the present study, Smad3 deletion had a more profound effect on collagen deposition than αSMA expression (Fig. 4 and 5), consistent with reports of less fibrosis in a dimethylnitrosamine model of liver fibrosis using Smad3KO×ex8 mice(30). Smad3 KO mice have altered mechanotransduction properties due, in part, to changes in ECM production(2), which are supported by in vitro studies demonstrating that Smad3 regulates cytoskeletal organization(55). Not surprisingly, the role of Smad3 in fibrosis or cirrhosis also depends on the type of fibrotic injury(28), and possibly the underlying etiology in human fibrosis.

It is interesting to note that genetic Smad3 deletion altered the expression of Ctgf, but not Tgfβ in PTg livers. In this model it is surprising that Tgfβ expression was not significantly decreased in PTg/KO mice, because this profibrotic molecule is regulated in an autocrine manner(14, 43), and thus would have been expected to be ‘downstream’ of Smad3. Our studies show that collagen levels decreased despite unchanged levels of Tgfβ gene expression. In our studies, decreased Ctgf expression correlated with decreased collagen gene expression and collagen deposition, suggesting that CTGF is a key fibrogenic regulator that is dependent on Smad3. However, recent studies have demonstrated that Ctgf induction is Smad3-independent, a relationship that may depend on the cell type or injury model that is used(19, 20, 33).

In vivo Smad2 and Smad3 have independent functions as revealed by genetic deletion studies(62). Deletion of Smad2 is embryonic lethal, while Smad3 deletion is permissive during development, regardless of the targeting construct(10, 62, 65). Similarly, Smad3 has separate, non-overlapping functions from Smad2 in hepatic fibrogenesis and HSC activation. In hepatocytes, Smad2 represses cell growth after injury, while Smad3 blocks apoptosis(26). In the present studies, we did not find evidence that Smad2 compensated for loss of Smad3. Thus, Smad2 and Smad3 regulate different physiological functions despite being 87% similar in amino acid sequence.

Smad3 deletion did not fully reverse effects of PDGF-C overexpression, however. This result was not unexpected as TGFβ stimulates a number of intracellular signaling pathways that are Smad-independent(11, 34, 38). Smad3-independent pathways that play key regulatory roles in HSC activation(21, 59) include PDGF receptor-mediated(1, 5), and extracellular fibular collagen and ECM signaling pathways.

Investigation of TGFβ signaling pathways in vitro is complicated by the evolving phenotype of primary HSCs when cultured(32). Isolated HSC become ‘activated’ when disaggregated from the liver and cultured, resulting in changes in Smad2 and Smad3 expression, which impacts their phenotype(32, 55). HSC expression of PDGF ligand and receptor depends on the degree of injury in the liver at the time of isolation, and on the length of
time in culture(6). In our study, deletion of Smad3 resulted in decreased proliferation and thymidine incorporation in HSCs. Previously published studies with Smad3 KO cells demonstrated dramatic effects of TGFβ on cell proliferation where MFB proliferation was enhanced after serum or PDGF-BB treatment when Smad3 (exon1) was deleted(51). In contrast, we observed slower cell growth and decreased proliferation after PDGF-CC treatment (Fig. 3). It is important to note that in vitro phenotypes with Smad2 and Smad3 null cells do not recapitulate in vivo phenotypes from mice that lack Smad2 and Smad3 (43:Verrecchia, 2001 #1309, 64). For example, Smad2 compensates for Smad3 in vitro, which is not seen during development.

The major cell types in the liver produce and respond to TGFβ ligands in a manner that is dependent on degree of inflammation and stage of disease. For example, TGFβ functions as a cell cycle inhibitor consistent with its role as a terminator of liver regeneration, in hepatocytes when inflammation and fibrosis are not apparent. HSCs produce collagen and ECM. In the context of hepatic inflammation and injury, most liver cells produce Tgfβ, including activated HSCs, hepatocytes and Kupffer cells(24). Cell-specific autocrine and paracrine consequence(s) of myofibroblasts-derived versus Kupffer-derived Tgfβ has not yet been explored. As TGFβ signaling is cell-specific and context dependent(37), we do not know whether cell specific deletion of Smad3 would yield the same results as systemic deletion.

An unanswered question from our studies is whether loss of TGFβ/Smad3 signaling would significantly delay hepatocellular carcinogenesis in PTg mice. A long-standing belief is that decreasing fibrosis will delay the onset of tumor development as cirrhotic livers hold the highest risk factor for developing HCCs in humans. As development of HCCs is preceded by severe fibrosis in PTg mice, a logical extension of this study would be to analyze tumorigenesis. However, few Smad3 KO mice survived beyond 5 months of age, and most died between 3 and 4 months of age. Smad3 KO mice were smaller than WT or PTg littermates and presented with a range of health issues, including malocclusion, mandible abscesses(63), penile prolapse, and cachexia (data not shown). High morbidity and early mortality has been reported for all Smad3 KO strains, independent of the targeting construct(10, 62, 65) Smad3 KO mice with exon 8 deletion on mixed 129 backgrounds were smaller than their littermates and had higher incidence of inflammatory lesions. In the present study, offspring resulting from Smad3 KOex8mice crossed with PDGF-C Tg mice (i.e. PTg/KO mice) had similar mortality as mice that only lacked the Smad3 allele. While it appears that the PDGF-C transgene does not appear to modify the effects of the systemic deletion of Smad3, the high mortality of these mice precluded analysis of hepatocellular carcinogenesis, which develops around 6 to 7 months of age. Thus, it remains to be determined whether attenuation of fibrosis by blocking TGFβ signaling via Smad3 would result in decreased incidence or prevalence of HCC in this model.

The present study, along with many other studies, provides strong evidence that
targeting TGFβ signaling in chronic liver disease would ameliorate liver fibrosis or cirrhosis if selective therapies could be developed. This anti-fibrotic approach continues to be revised in light of recent studies and previous attempts to block TGFβ signaling in pre-clinical models(49). The challenge of blocking TGFβ signaling is that this cytokine has cell-specific roles that continually change depending on disease type and stage, which may lead to unanticipated side effects(4). Moreover, our studies suggest that PDGF-C regulates TGFβ signaling pathways and that block of both these signaling pathways may be required for effective long-term therapy for chronic liver disease and prevention of HCC.

Acknowledgements. We thank Dr. Anita Roberts for providing the Smad3 KO mice, Drs. Anthony Parks, William Grady, Nelson Fausto and Deb Gilbertson for insightful discussions and valuable comments on the manuscript.

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Table 1. Genetic Analysis of Offspring Obtained from Smad3 KO;PDGF-C Tg Breeding Pairs.

<table>
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<th>Genotypes</th>
<th>Smad3&lt;sup&gt;WT/WT&lt;/sup&gt; PDGF-&lt;sup&gt;C&lt;/sup&gt; Tg&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>Smad3&lt;sup&gt;ex8/WT&lt;/sup&gt; PDGF-&lt;sup&gt;C&lt;/sup&gt; Tg&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>Smad3&lt;sup&gt;ex8/ex8&lt;/sup&gt; PDGF-&lt;sup&gt;C&lt;/sup&gt; Tg&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>Smad3&lt;sup&gt;WT/WT&lt;/sup&gt; PDGF-&lt;sup&gt;C&lt;/sup&gt; WT&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>Smad3&lt;sup&gt;ex8/WT&lt;/sup&gt; PDGF-&lt;sup&gt;C&lt;/sup&gt; WT&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>Smad3&lt;sup&gt;ex8/ex8&lt;/sup&gt; PDGF-&lt;sup&gt;C&lt;/sup&gt; WT&lt;sup&gt;WT&lt;/sup&gt;</th>
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<tr>
<td><strong>Expected number&lt;sup&gt;2&lt;/sup&gt;</strong></td>
<td>26</td>
<td>51</td>
<td>26</td>
<td>26</td>
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<td>53</td>
<td>4</td>
<td>31</td>
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<td>6</td>
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<tr>
<td><strong>Expected percentage&lt;sup&gt;2&lt;/sup&gt;</strong></td>
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<td>25</td>
<td>12</td>
<td>12</td>
<td>25</td>
<td>12</td>
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<tr>
<td><strong>Actual percentage</strong></td>
<td>22</td>
<td>26</td>
<td>2</td>
<td>15</td>
<td>32</td>
<td>3</td>
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Chi-square analysis was performed on genotypes from 206 mice from four different sets or breeders to generate a chi-squared value of 15.59. As this number is greater than 11.07 (the value for five degrees of freedom), this analysis indicates that fewer Smad3 null mice are born than expected.

<sup>1</sup> Genotypes were obtained from pups born to breeder pairs consisting of female Smad3<sup>ex8/WT</sup>;PDGF-<sup>C</sup> WT<sup>WT</sup> and males Smad3<sup>ex8/WT</sup>;PDGF-C<sup>TgWT/WT</sup> mice.

<sup>2</sup> These would be the expected numbers of pups of each genotype based on Mendelian ratios.
Figure Legends

Figure 1. Evidence of TGFβ signaling in livers of PDGF-C Tg (PTg) mice. Analysis of gene expression (A), protein (B) and phosphorylated Smad2/3 (C) was performed using livers from PTg mice compared to WT mice (8-9 weeks of age, n=6-7). (A) TGFβ1 receptor (Tgfβr1) (A.1), Smad3 (A.2) and Smad7 (A.4) gene expression is higher in PTg mice, while Smad2 (A.3) expression did not change. Total RNA was prepared from livers of PTg or WT littermates. Real time PCR was performed to determine mRNA levels of Tgfβr1, Smad2, Smad3, and Smad7 as described in the Materials and Methods. Data are represented as fold change compared to a WT animal. (B) Immunoblot analysis of Smad2 and Smad3 indicate that Smad3 is elevated in PTg mice liver tissue, while Smad2 levels were similar to WT mice. The bar graph represents the average of densitometry value for two animals after normalizing to GAPDH levels. (C) Phosphorylated Smad2/3 is present in hepatocyte nuclei, and NPCs in livers from PTg (C.2 and C.4), but not WT (C.1 and C.3) mice. Immunohistochemical analysis was performed on 2-month (C.1 and C.2), and 4.5-month old (C.3 and C.4) mice as described in Materials and Methods. Both stained (black arrowheads) and unstained (white arrowheads) hepatocyte nuclei can be seen in the same field. Scale bar represents 50 microns.

Figure 2. Deletion of Smad3 attenuates PDGF-C-induced effects in the liver. PTg mice were intercrossed to Smad3 KOex8/ex8 and liver tissue from four groups (wild-type (WT/WT), Smad3 KO (WT/KO), PDGF-C Tg (PTg/WT), and PDGF-C Tg; Smad3 KO (PTg/KO) were analyzed for (A) Smad2 and Smad3 protein levels, (B) circulating levels of human PDGF-CC, (C) liver weight/body weight ratio, (D) spleen weight/body weight ratio, (E) hepatocyte and (F) NPC proliferation (2-5 months of age, n=4-5). Mann Whitney. (*), P < 0.05; (**), P <0.01; (***) , P < 0.001.

Figure 3. Effects of Smad3 deletion on ³H-thymidine incorporation in HSCs in vitro. Effect of Smad3 deletion in HSCs was evaluated using primary HSCs isolated from both WT and Smad3 KO (KO) mice in serum containing media (A) and after PDGF-CC stimulation (B). (A) Isolated primary Smad3 null HSCs grow more slowly than WT HSCs. Isolated cells were plated in serum-containing media, and cells were counted on days 0, 2, 4, 6, 9, 11. On day 11, WT HSC cultures were confluent, Smad3 KO cultures were not. Each line represents HSCs from individual mice. (B) PDGF-CC-induced HSC ³H-thymidine incorporation is significantly decreased in Smad3 null HSCs. Isolated HSCs were cultured for seven days, serum-starved and then either treated with PDGF-CC (25 ng/mL) or left untreated. ³H-thymidine incorporation was used to assess cell proliferation. These data are representative of two independent experiments.
**Figure 4.** PDGF-C Tg;Smad3 KO mice have decreased collagen deposition compared to PTg mice. Liver tissue from PDGF-C Tg and PDGF-C Tg;Smad3 KO mice was stained for collagen deposition using Masson’s trichrome (A and C, 4 months of age) and picrosirius red (B and D, 3 months of age). The extensive collagen deposition seen in PDGF-C Tg mice (A and B), is decreased in PDGF-C Tg;Smad3 KO mice. Scale bar 50 microns.

**Figure 5.** Fibrosis is significantly decreased in PDGF-C Tg;Smad3 KO mice. Liver tissue from WT, Smad3 KO, PTg and PTg/KO mice was stained for collagen deposition using picrosirius red staining and morphometric analysis done as described in Materials and Methods. (2-5 months of age, n=4-5). (*) P < 0.05; (**) P <0.01.

**Figure 6.** Loss of Smad3 blunts induction of genes involved in collagen gene expression, but not genes associated with HSC activation. RNA was isolated from liver tissue from WT, Smad3 KO (KO), PDGF-C Tg (Tg) and PDGF-C Tg;Smad3 KO (Tg/KO) mice (2-5 months of age, n=4-5). Changes in gene expression were determined using real-time RT PCR normalized to Gapdh as described in the Materials and Methods. Average delta Ct values +/- SEM are shown for each gene analyzed, and data are displayed for WT (open bars), KO (speckled bars); Tg (black bars), and Tg/SKO (hatched bars) mice. (A) Genes whose expression are decreased by loss of Smad3 (Smad3-dependent genes), and (B) genes that did not show a significant difference when Smad3 is deleted (i.e. Tg compared to Tg/KO mice) are shown. (*), P < 0.05; (**), P <0.01.

**References**


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A. Cell number (1.8 cm² grid) over time (day) for different genotypes:
- WT1
- WT2
- S3KO 1
- S3 KO 2
- S3 KO 3

B. DNA synthesis (3H-Thymidine, CPM) with Smad3 and PDGF-CC:
- WT
- KO
- WT (PDGF-CC +)
- KO (PDGF-CC +)

* indicates a significant difference.
A. Smad3-dependent genes

Gene expression (relative to GAPDH)

- Acta2
- Ctgf
- Col1a1
- Col1a2
- Col1a4

Mouse Genotype:
- WT
- KO
- TG
- TG/KO

B. Smad3-independent genes

Gene expression (relative to GAPDH)

- Pdgfra
- Pdgfrb
- Tgfb1
- Crbp1

Mouse Genotype:
- WT
- KO
- TG
- TG/KO

* indicates statistical significance.