The pituitary tumour-transforming gene 1/delta-like homologue 1 pathway plays a key role in liver fibrogenesis

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Abstract

Background and Aims: PTTG1 is almost undetectable in adult livers but is highly expressed in hepatocarcinoma. While little is known about its involvement in liver fibrosis, PTTG1 expression is associated with DLK1. We assessed the role of the PTTG1/DLK1 pathway in fibrosis progression and the potential therapeutic effect of PTTG1 silencing in fibrosis.

Methods: Pttg1 and Dlk1 were studied in liver and isolated cell populations of control and fibrotic rats and in human liver biopsies. The fibrotic molecular signature was analysed in Pttg1+/− and Pttg1+/− fibrotic mice. Finally, Pttg1 silencing was evaluated in rats as a novel antifibrotic therapy.
**Lay Summary**

PTTG1 and DLK1 transcription are increased in rats and patients with hepatic cirrhosis. PTTG1 is involved in fibrotic extracellular matrix remodeling and its silencing decreases portal hypertension and alleviates fibrosis progression.

1 | INTRODUCTION

Cirrhosis is a major determinant of morbidity and mortality and pre-disposes to hepatic failure and liver cancer. Halting the progression of fibrosis to cirrhosis is considered as a foremost goal in patients with liver disease. Anti-inflammatory agents, arresting hepatic stellate cells (HSC) activation substances, renin-angiotensin system inhibitors, cannabinoid receptor antagonists, hepatoprotective late cells (HSC) activation substances, renin-angiotensin system of fibrosis to cirrhosis is considered as a foremost goal in patients disposes to hepatic failure and liver cancer. Halting the progression of liver fibrosis.

**Results:** Pttg1 and Dlk1 mRNA selectively increased in fibrotic rats paralleling fibrosis progression. Serum DLK1 concentrations correlated with hepatic collagen content and systemic and portal haemodynamics. Human cirrhotic livers showed greater PTTG1 and DLK1 transcript abundance than non-cirrhotic, and reduced collagen was observed in Pttg1 Pttg1−/− mice. The liver fibrotic molecular signature revealed lower expression of genes related to extracellular matrix remodeling including Mmp8 and 9 and Timp4 and greater eotaxin and Mmp13 than fibrotic Pttg1+/− mice. Finally, interfering Pttg1 resulted in reduced liver fibrotic area, lower α-Sma and decreased portal pressure than fibrotic animals. Furthermore, Pttg1 silencing decreased the transcription of Dlk1, collagens I and III, Pdgfrβ, Tgfrβ, Timp1, Timp2 and Mmp2.

**Conclusions:** Pttg1/Dlk1 are selectively overexpressed in the cirrhotic liver and participate in ECM turnover regulation. Pttg1 disruption decreases Dlk1 transcription and attenuates collagen deposition. PTTG1/DLK1 signalling is a novel pathway for targeting the progression of liver fibrosis.

KEYWORDS
extracellular matrix, fibrosis, gene therapy, liver, siRNA

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PTTG1 is the pituitary tumour-transforming gene (PTTG1) is the index mammalian securin.5 PTTG1 is overexpressed in a variety of cell lines including hepatocellular carcinoma (HCC).6 It encodes a multifunctional protein involved in the regulation of faithful chromatid segregation during mitosis, DNA repair, apoptosis, metabolism and gene transcription.7 Interestingly, PTTG1 modulates extracellular matrix (ECM) turnover regulating several matrix metalloproteinases (MMPs).8,9 Despite overexpression of PTTG1 in liver biopsies from patients with HCC, very little data are available on its expression in preneoplastic conditions such as advanced liver fibrosis and cirrhosis. This is particularly striking as several factors induce PTTG1 expression, including estrogens, fibroblast growth factor, insulin, insulin growth factor-1 and hepatocyte growth factor.20–22 all increased under conditions of chronic liver injury.11-14 Moreover, micro-environmental hypoxia occurring in damaged hepatic tissue could also regulate PTTG1 expression through the hypoxia-inducible factor 1.15 PTTG1 also acts to regulate growth factors, angiogenesis and exhibits transforming activity in vitro and in vivo.16,17 Furthermore, hepatic PTTG1 expression is upregulated after partial hepatectomy and has been proposed as a new marker of proliferation in liver regeneration.18 These findings support the exploration of whether PTTG1 could contribute to the activation of fibroproliferative processes in liver disease. In addition, delta-like homologue 1 (DLK1) was identified as one of the most abundantly expressed PTTG1 targets.19 The DLK1 gene encodes a single-pass transmembrane protein that belongs to a family of epidermal growth factor (EGF) repeat-containing proteins.20 DLK1 is a non-canonical ligand of Notch receptors that mediate a metabolic shift from lipid storage to peripheral lipid oxidation in adipocytes, participate in differentiation processes and behave as a growth factor.21 It consists of six EGF-like tandem repeats, a juxtamembrane region with a tumour necrosis factor-alpha converting enzyme (TACE)-mediated cleavage site, a transmembrane domain and a short intracellular tail.22 DLK1 can act as both transmembrane and soluble protein. DLK1 membrane-proximal cleavage by TACE results in the release of the EGF-like
extracellular region, a large soluble product of 50 kDa. This form has a similar function inhibiting adipocyte differentiation to that of the full-length membrane-associated protein, but since it is soluble it can act in an autocrine and paracrine manner. Moreover, both PTTG1 and DLK1 genes show concomitant expression in human fetal liver, placenta and different carcinomas, including pituitary adenoma, breast adenocarcinoma and neuroblastoma.\textsuperscript{19} Given this background, we aimed to explore the hypothesis that PTTG1/DLK1 signalling should play a central role in the activation of the fibrogenic process in liver disease.

2 | MATERIALS AND METHODS

2.1 | Induction of hepatic cirrhosis in rats

This study was performed in control (n = 32) and male Wistar rats with different degrees of fibrosis (n = 77) (Charles-River, Saint Aubin Les Elseuf, France). Fibrosis was induced by repetitive carbon tetrachloride (CCl\(_4\)) inhalation.\textsuperscript{23} The rats were fed ad libitum with standard chow and water containing phenobarbital (0.3 g/L), as drinking fluid. Animals were exposed to a CCl\(_4\) atmosphere twice a week, starting with 0.5 minutes for three sessions. Afterwards, the duration was increased to 1, 2, 3, 4 and 5 minutes until the end of the investigation. To induce variable degrees of hepatic fibrosis CCl\(_4\)-treated rats were studied at the 8th, 13th, 16th and 19th week after starting the fibrosis induction protocol. Control rats were studied following similar periods of phenobarbital administration. When scheduled, animals were anaesthetised and a haemodynamic study was performed. Afterwards, a blood sample was obtained and animals were sacrificed by isoflurane overdose (Forane, Abbott Laboratories S.A., Madrid, Spain). Organ samples were snap-frozen or fixed in 10% buffered formalin.

2.2 | Induction of fibrosis in mice

This study was performed in fibrotic and control male Pttg1 wild-type (Pttg1\(^{+/+}\)) and knock out (Pttg1\(^{−/−}\)) mice. Pttg1\(^{−/−}\) mice with C57BL/6 genetic background were provided by Dr Shlomo Melmed and the origin of these mice has been described previously.\textsuperscript{24} Fibrosis was induced in Pttg1\(^{+/+}\) (n = 4) and Pttg1\(^{−/−}\) (n = 7) by i.p. injection of CCl\(_4\) (1 ml CCl\(_4\)/kg of body weight [bwt], previously diluted 1:8 vol/vol in corn oil) three times a week for 4 weeks. All animals were kept under constant temperature and humidity in 12 hours controlled dark/light cycle, and they were fed ad libitum on a standard pellet diet.

2.3 | In vivo Pttg1 interference

A group of fibrotic rats randomly received i.v. Pttg1 small-interfering RNA (siRNA, assay ID s133880, 0.25 mg/kg/dose bwt, n = 6) or scrambled siRNA (Ambion in vivo negative control No. 1, n = 6) as the negative control (C\textsuperscript{−}siRNA) every 10 days from the 9th to the 13th week after starting the fibrosis induction protocol. In vivo transfection was performed using Invivofectamine 3.0 kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer’s instructions. Six control rats were also included. Rats were studied in the 14th week.

2.4 | Statistical analysis

Quantitative data were analysed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) and statistical analysis of the results was performed by unpaired Student’s t test, one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test or Kruskal-Wallis test with Dunn post hoc test when appropriate. Correlations between the variables studied were analysed with Pearson two-tailed test. Results are expressed as mean ± SE and considered significant with P < 0.05.

Additional materials and methods are provided in the Supplementary material section.

3 | RESULTS

3.1 | Fibrosis quantification and staging

The liver of rats treated with CCl\(_4\) showed macroscopic finely granulated surfaces. According to the time of CCl\(_4\) exposure, we observed progressive ECM accumulation, evolving from a light deposition, mainly in the portal area, to numerous and thicker septa in those animals submitted to longer CCl\(_4\) exposure periods. Most animals exposed to the toxin for the longest periods of time developed cirrhosis. Consequently, rats were staged according to the percentage of fibrotic area with respect to the total area of the liver biopsy: mild and moderate fibrosis was defined when the percentage of fibrotic area <6% (n = 6), severe fibrosis 6%–11%, (n = 8) and cirrhosis >11% (n = 11). Control rats (n = 13) displayed no appreciable alterations in liver histology. Figure 1A shows representative Sirius red staining from a control liver, a liver with mild/moderate fibrosis, a liver with severe fibrosis and a cirrhotic liver. Fibrotic/cirrhotic rats had important alterations in liver function tests, which were more pronounced in cirrhosis (Table S1).

3.2 | Hepatic Pttg1 and Dlk1 mRNAs parallel the intensity of liver fibrosis and selectively occurs in this organ

Progression of liver fibrosis was associated with a concomitant increase in Pttg1 mRNA expression (Figure 1B). Pttg1 expression significantly increased in rats with severe fibrosis and reached maximum levels in rats with cirrhosis. Interestingly, Pttg1 transcript was selectively detected in the liver of cirrhotic animals, but not in the
spleen, lungs, kidneys, heart, aorta or brain (Figure 1C). Pttg1 mRNA abundance was also assessed by droplet digital PCR (ddPCR). Results were in line with those obtained in real-time PCR (RT-PCR) experiments. The liver of cirrhotic rats showed a much higher abundance of Pttg1 transcripts (411 ± 67 copies/μl) than that found in control livers (20 ± 2 copies/μl, P < 0.001). In contrast, with the exception of heart (7 ± 1 vs 28 ± 2 copies/μl, P < 0.05) no differences were found between spleen (614 ± 94 vs 947 ± 172 copies/μl), kidney (27 ± 4 vs 38 ± 5 copies/μl), lung (74 ± 8 vs 87 ± 5 copies/μl) aorta (4 ± 1 vs 27 ± 12 copies/μl) and brain (17 ± 3 vs 21 ± 1 copies/μl) of cirrhotic and control rats. The pattern expression of Pttg1 was paralleled by a similar profile for Dlk1 mRNA (Figure 1B). Dlk1 mRNA abundance progressively increased, the lowest levels observed in rats with mild/moderate fibrosis, the highest in cirrhotic rats. Indeed, Dlk1 activation was selectively detected in the cirrhotic liver but not in other assessed organs (Figure 1C).

3.3 | Pttg1 and Dlk1 are mainly expressed in hepatic parenchymal tissue

To identify the cellular source of altered expression of both Pttg1 and Dlk1 in hepatic tissue, we isolated primary cells from the liver of cirrhotic and control rats. Both, Pttg1 and Dlk1 exhibited low or almost negligible mRNA expression in different control cell types (Figure 2A). By contrast, marked Pttg1 mRNA abundance was
observed in three types of liver cells isolated in cirrhotic rats, the highest abundance being found in HSC (Figure 2A). This was paralleled by striking activation of $Dlk1$ mRNA but was largely observed in hepatocytes (HEP) (Figure 2A). In an attempt to further delineate the relative contribution of HEP and HSC to the acute increase in $Pttg1$ and $Dlk1$ in cirrhotic liver, we next measured the absolute concentration of these transcripts in the isolated cells. In line with the RT-PCR results, the absolute $Pttg1$ mRNA values were similar in both types of cells (HEP: $125 \pm 7$ copies/μl, HSC: $131 \pm 3$ copies/μl), whereas $Dlk1$ mRNA values were lower in HSC ($80 \pm 11$ copies/μl) than in HEP ($154 \pm 9$ copies/μl). Overactivation of the PTTG1/DLK1 axis in human cirrhosis was further confirmed. Paralleling the increased abundance of collagen I alpha 1 ($COL1α1$) messenger, higher expression of both $PTTG1$ and $DLK1$ mRNA was observed in samples derived from cirrhotic patients in comparison to non-cirrhotic biopsies. Next, we performed histological immunolocalization of PTTG1 and DLK1 in the liver of cirrhotic and control rats. Both proteins were almost undetectable in control samples. However, in cirrhotic livers, they were clearly identified either in the parenchymal area or close to the portal tracts and fibrous septa (Figure 2C). Additionally, DLK1 expression clearly differs from that of other well established profibrogenic substances, since DLK1 hepatic protein content only exhibited a clear relationship with fibrosis intensity (Figure 2D).

### 3.4 Serum DLK1 values rise in parallel with liver fibrosis and correlate with hemodynamics

Advanced progression of liver fibrosis was associated with a parallel increase in circulating levels of DLK1, showing a significant increase in rats with cirrhosis ($53.13 \pm 5.66$ ng/ml, $P < 0.001$) (Figure 3A). A close direct relationship between DLK1 and hepatic collagen content was found in $CCl_4$-treated rats ($r = 0.74; P < 0.001$) (Figure 3B). Animals with cirrhosis showed frank hypotension (mean arterial pressure [MAP]: $87 \pm 13$ mm Hg, $P < 0.001$) as compared with control rats (MAP: $123 \pm 2$ mm Hg).
MAP inversely correlated with serum DLK1 in CCl$_4$-treated rats ($r = -0.69$, $P < 0.001$) (Figure 3C). Furthermore, serum concentration of DLK1 also depicted a direct relationship with the degree of portal hypertension in fibrotic/cirrhotic animals ($r = 0.41$, $P < 0.05$) (Figure 3D).

3.5 Fibrosis is significantly attenuated in Pttg1$^{-/-}$ mice

CCL$_4$-treated Pttg1$^{-/-}$ mice had mild/moderate fibrosis mainly characterized by perivenular and perilportal deposition with incipient development of the portal and venular septa, ending blindly in the parenchyma; whereas Pttg1$^{-/-}$ mice displayed thinner septa and more preserved hepatic parenchyma (Figure 4A) than Pttg1$^{+/+}$ animals. These findings were confirmed by morphometric analysis in which Pttg1$^{-/-}$ mice showed a significantly reduced percentage of fibrosis area than sections of Pttg1$^{+/+}$ mice (Figure 4B). This attenuation in liver fibrosis was also associated with an almost 50% reduction in Dlk1 mRNA expression. In fact, whereas fibrotic Pttg1$^{+/+}$ mice showed a $55.7 \pm 5.0$-fold change increase in Dlk1 mRNA overcontrol Pttg1$^{-/-}$ mice, these figures were of a $30.9 \pm 7.5$-fold change increase in Pttg1$^{-/-}$ fibrotic mice.

3.6 Expression pattern of fibrogenesis-related genes in Pttg1$^{-/-}$ mice

Further insight on the effects of Pttg1$^{-/-}$ in the liver of CCL$_4$-treated mice was obtained by determining the mRNA expression of 86 genes involved in the fibrogenic process. Table 1 shows all the genes showing a 1.5-fold or greater change in expression with Pttg1$^{+/+}$ control or CCL$_4$-treated mice. Regardless of the moderate hepatic collagen deposition observed in Pttg1$^{+/+}$ mice treated with CCL$_4$, these animals showed a clear fibrotic molecular signature. Actually, 24 genes were significantly upregulated, including Acta2 and Grem1 which encode proteins involved in HSC activation and epithelial to mesenchymal transition, genes related to ECM and adhesion molecules (Col1a2, Col3a1, Mmp1a, Mmp9 and Plau) and several genes related to inflammation (Il1b, Il1f, Ilk, It10, Tnf, Ccl2 and Cxcr4), growth (Agt) and signal transduction (Inhbe, Smad3, Stat6, Smad6, Tgfb1, Tgfb2, Tgfr2, Thbs1 and Thbs2) compared to control Pttg1$^{+/+}$ mice.

A 1.5-fold or greater change in expression with $P < 0.05$ was considered statistically significant in comparing fibrotic Pttg1$^{+/+}$ vs Pttg1$^{-/-}$ mice. In addition to reducing the mRNA expression of Acta2 (gene encoding $\alpha$-smooth muscle actin protein), the lack of Pttg1 inhibited the expression of several genes involved in ECM turnover including Mmp8, Mmp9 and Timp4 (Figure 4C). We also observed a significant increase in Ccl11 and Mmp13. The former encodes eotaxin a chemokine that has been described to be upregulated in senescent HSC$^{26}$ whereas the latter encodes for a metalloprotease involved in the degradation of a fibrotic liver matrix.$^{27}$

3.7 Assessment of Pttg1 siRNA in cultured rat hepatocytes and fibrotic rats

To investigate the efficacy and duration of gene silencing in cultured rat hepatocytes, we transfected CC-1 cells. Following treatment,
Pttg1 mRNA was significantly lower than that in the siRNA C− group at 24, 48 and 72 hours (Figure 5A). These results indicate that Pttg1 siRNA effectively suppresses Pttg1 expression in rat cultured hepatocytes. Pttg1 siRNA was also effective at silencing the enhanced expression of Pttg1 mRNA in fibrotic rats (Figure 5B). In fact, whereas fibrotic rats treated with scrambled siRNA displayed approximately 15 times higher levels of Pttg1 mRNA than control animals, an abundance of this transcript in the liver of fibrotic rats receiving Pttg1 siRNA was not different from that found in healthy animals. In addition to silencing hepatic Pttg1 mRNA, administration of Pttg1 siRNA also inhibited hepatic Dlk1 mRNA expression in fibrotic rats (Figure 5B).

3.8 | Effect of Pttg1 siRNA on liver histology, portal pressure and profibrogenic genes in fibrotic rats

Fibrotic rats treated with C− siRNA showed initial stages of the characteristic pattern of perivenular and periportal deposition of connecting tissue with development of portal-to-portal septa and evidence of architectural distortion resulting in micronodular fibrosis (Figure 6A). However, biopsies obtained from fibrotic rats treated with Pttg1 siRNA displayed less remarkable architectural alterations, with thinner septa, and more preserved hepatic parenchyma. This was confirmed by morphometric analysis of Sirius red-stained sections (Figure 6B). This abrogation of fibrosis was consistently observed in all animals exposed to Pttg1 silencing. Similar results were found when staining alpha 2 smooth muscle actin (α-SMA). We detected α-SMA as linear staining in the portal tracts and fibrous septa of both groups of fibrotic rats (Figure 6A). Staining was more diffuse in rats receiving Pttg1 siRNA than in C− siRNA. In line, Pttg1 siRNA also showed significantly reduced portal hypertension than fibrotic animals receiving C− siRNA (Figure 6B). Furthermore, Pttg1-silenced animals significantly decreased hepatic mRNA expression of Tnfα compared to fibrotic rats.

As anticipated, Col1α2 and Col3α1 mRNA was significantly increased in fibrotic rats treated with C− siRNA. Consistently, we also observed activation of key genes involved in profibrogenic mechanisms, such as Tgfβr1 and Pdgfrβ (Figure 6C), and an altered balance of MMPs and TIMPs, specifically, increased transcription of Mmp2, Mmp9, Timp1 and Timp2 (Figure 6D). In line with previous results, administration of Pttg1 siRNA resulted in a significantly lower abundance of Col1α2, Col3α1, Mmp2, Timp1 and Timp2 transcripts (Figure 6C,D). Furthermore, Tgfβr1 and Pdgfrβ expression appeared also markedly attenuated, indicating that Pttg1 silencing effectively abrogates profibrogenic activity in CCl4-induced fibrosis.
3.9 Effect of Pttg1 siRNA on serum markers of liver function

Pttg1 siRNA treatment was associated with a tendency towards normalization of most systemic indicators of liver function (Table 2). Actually, aspartate aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, total bilirubin and triglycerides were found to be near normal. Overall, these results support the protective effects on hepatic function resulting from Pttg1 mRNA silencing in rats with experimental fibrosis.

### TABLE 1 Hepatic mRNA expression of genes involved in pathogenic mechanisms of liver fibrosis showing 1.5-fold or greater regulation between control Pttg1+/+(n = 4) and fibrotic Pttg1−/−(n = 4) mice

| Gene symbol | Fold regulation | Gene symbol | Fold regulation |
|-------------|----------------|-------------|----------------|
| **Fibrosis** |                |             |                |
| Acta2 | 1.92** | Bcl2 | 2.35 |
| Grem1 | −2.35† | Fasl | 1.78 |
| **Extracellular matrix and cell adhesion molecules** | | | |
| Col1a2 | 4.72*** | Mmp9 | 4.44** |
| Col3a1 | 3.79*** | Mmp13 | 5.59 |
| Lox | 2.95 | Mmp14 | 2.42 |
| Itga2 | 2.38 | Plat | 7.52 |
| Itgb3 | 1.57 | Plau | 4.50* |
| Itgb5 | 1.57 | Serpine1 | 9.35 |
| Mmp1a | 4.54** | Timp1 | 2.84 |
| Mmp2 | 8.51 | Timp2 | 2.06 |
| Mmp3 | 1.55 | Timp3 | 1.86 |
| Mmp8 | 4.55 | Timp4 | 1.96 |
| **Inflammatory cytokines and chemokines** | | | |
| Ccl3 | 7.58* | Il1a | 2.98** |
| Ccl11 | −1.75 | Il1b | 2.69* |
| Ccr2 | 4.35 | Il4 | −1.56 |
| Cxcr4 | 3.44* | Il5 | −1.61 |
| Ifng | 2.38 | Ilk | 1.53** |
| Il10 | 4.43* | Tnf | 4.64* |
| Il13ra2 | 1.82 |                |                |
| **Growth factors** | | | |
| Agt | 1.60** | Egf | 1.73 |
| Ctgf | −1.65 | Pdgfa | 1.72 |
| Edn1 | 1.52 | Pdgfb | 2.43 |
| **Signal transduction** | | | |
| Cav1 | 1.51 | Tgb81 | 2.04** |
| Inhbe | 2.85 | Tgb2 | 2.67 |
| Myc | 2.69 | Tgb3 | 2.69 |
| Smad3 | 1.83* | Tgb3r1 | 1.79* |
| Smad6 | 2.17* | Tgb2r2 | 2.37* |
| Smad7 | 1.91 | Thbs1 | 3.01 |
| Stat6 | 1.69* | Thbs2 | 2.98 |
| **Epithelial-to-mesenchymal transition** | | | |
| Akt1 | 1.63 | | |

Abbreviations: mRNA determined by Acta2. Alpha 2 smooth muscle actin; Agt, angiotensinogen; Akt1, AKT serine/threonine kinase 1; Bcl2, B-cell lymphoma 2; Cav1, caveolin 1; Ccl3, C–C Motif chemokine ligand 3; Ccl11, C–C motif chemokine ligand 11; Ccr2, C–C motif chemokine receptor 2; Col1a2, collagen type I Alpha 2 Chain; Col3a1, collagen type III Alpha 1 Chain; Ctgf, cellular communication network factor 2; Cxcr4, C–X–C motif chemokine receptor 4; Edn1, Endothelin 1; Egf, epidermal growth factor; Fasl, tumour necrosis factor receptor superfamily, member 6 Ligand; Grem1, Gremlin 1; Ifng, interferon gamma; Il1a, Interleukin 1 Alpha; Il1b, Interleukin 1 Beta; Il4, Interleukin 4; Il5, Interleukin 5; Il10, Interleukin 10; Il13ra2, Interleukin 13 Receptor Subunit Alpha 2; Ilk, Integrin Linked Kinase; Inhbe, Inhibin Subunit Beta E; Itga2, Integrin Subunit Alpha 2; Itgb3, Integrin Subunit Beta 3; Itgb5, Integrin Subunit Beta 5; Lox, Lysyl Oxidase; Mmp1a, Matrix Metalloproteinase 1a; Mmp2, matrix metalloproteinase 2; Mmp3, matrix metalloproteinase 3; Mmp8, matrix metalloproteinase 8; Mmp9, matrix metalloproteinase 9; Mmp13, matrix metalloproteinase 13; Mmp14, matrix metalloproteinase 14; Myc, MYC proto-oncogene, BHLH transcription factor; Pdgfa, platelet-derived growth factor subunit A; Pdgfb, platelet-derived growth factor subunit B; Plat, placminogen activator, tissue type; Plau, placminogen activator,urokinase; Serpine1, serpin family E member 1; Smad3, SMAD family member 3; Smad6, SMAD family member 6; Smad7, SMAD family member 7; Stat6, signal transducer and activator of transcription 6; Tgb1, transforming growth factor-beta 1; Tgb2, transforming growth factor-beta 2; Tgb3, transforming growth factor-beta 3; Tgb3r1, transforming growth factor-beta receptor 1; Tgb2r2, transforming growth factor-beta receptor 2; Thbs1, thrombospondin 1; Thbs2, thrombospondin 2; Timp1, tissue inhibitor of metalloproteinase 1; Timp2, tissue inhibitor of metalloproteinase 2; Timp3, tissue inhibitor of metalloproteinase 3; Timp4, tissue inhibitor of metalloproteinase 4; Tnf, tumour necrosis factor. *P < 0.05; **P < 0.01; ***P < 0.001 vs control WT mice. Unpaired Student’s t test.

This investigation aimed to explore whether PTTG1/DLK1 signalling contributes to the activation of the fibroproliferative process in liver disease. In agreement with previous studies, Pttg1 mRNA was almost undetectable in healthy animals. In contrast, Pttg1 mRNA levels were markedly overexpressed in the liver of rats with hepatic fibrosis, reaching maximal abundance in cirrhotic rats. Moreover, only hepatic tissue of cirrhotic rats showed a significantly increased abundance of Pttg1 mRNA with respect to control animals. Paralleling Pttg1 results, Dlk1 mRNA expression markedly increased in fibrotic rats, with a close correlation with collagen deposition. Dlk1 transcript was also selectively overexpressed only in the liver. In addition, liver Dlk1 protein levels mirrored expression patterns of the cognate transcript. The lowest abundance was found in rats with mild fibrosis and the highest in cirrhotic rats. This clearly differs from the behaviour of other profibrogenic factors. Cell fractionation experiments showed increased Pttg1 and Dlk1 mRNA in HEP, HSCs and endothelial cells (ECs) in liver tissue of cirrhotic rats compared to controls. Furthermore, PTTG1 and DLK1 protein
staining in cirrhotic rats resulted in an intense, although topologically undefined, positive signal in the hepatic parenchyma, being more pronounced close to the portal tracts. Finally, on exploring the expression of PTTG1/DLK1 in the human liver we also observed that, whereas control livers showed negligible PTTG1 or DLK1 mRNA expression, samples from cirrhotic patients markedly overexpressed PTTG1 mRNA. To our knowledge, this is the first investigation demonstrating PTTG1 mRNA induction in human cirrhosis. In parallel, DLK1 was also increased in human samples in line with previous investigations showing that DLK1 is frequently upregulated in human HCC but rarely detected in adjacent non-cancerous liver tissue. Although, our findings indicate that the PTTG1/DLK1 pathway is relevant in the pathogenesis of liver fibrosis. This contention was further supported by results obtained in Pttg1 KO mice. Previous investigations performed in thioacetamide-induced fibrosis showed significantly weaker macromorphological signs of bridging fibrosis in Pttg1−/− in comparison to Pttg1+/− mice. This finding was further confirmed in the current study in CCL4-treated mice, indicating that the lack of Pttg1 attenuates the development of murine liver fibrosis. Subsequent gene expression analysis pointed toward disruption of ECM turnover as a major driver of this phenomenon. In fact, stringent analysis of alterations in gene expression induced by Pttg1−/− in fibrotic animals, considering only those genes showing both statistically and biologically significant downregulation, revealed a group of genes all involved in fibrogenesis including Mmp9, Mmp8, Timp4 and Acta2. Of interest, this occurred in the setting of diminished Myc mRNA expression, which encodes a nuclear phosphoprotein that regulates the transcription of numerous genes involved in the cell cycle, cell growth, differentiation, apoptosis, transformation, genomic stability and angiogenesis. Moreover, Pttg1 is a powerful activator of Myc, which suggest that this gene could play a central role in PTTG1 induced fibrosis signalling pathway. Our findings suggest that RNA-based therapy targeting Pttg1, such as siRNA, may prevent the development of liver fibrosis. siRNA-Pttg1-treated rats displayed significantly weaker macromorphological signs of liver fibrosis, a decrease in portal hypertension and a lesser amount of activated HSC. These results suggest that a reduction in the proportion of activated HSC is involved in the inhibition of liver fibrogenesis. Amelioration in portal pressure is most likely a consequence of the antifibrotic effect induced by Pttg1 siRNA administration. These results are also supported by a lower abundance of liver Col1α2 and Col3α1 mRNAs and some other markers of active fibrosis such as Tgfβ1 and Pdgfβ. It has been reported that interference of PTTG1 in ovarian epithelial tumour cells resulted in diminished expression and release of TGFβ, whereas increased expression of Pttg1 mRNA mirrored Tgfβ mRNA expression.

In our study Pttg1 mRNA interference tended towards lower levels of Tgfβ1, although without statistical significance. This is consistent with previous reports in fibrotic Pttg1 null mice in which hepatic Tgfβ mRNA was significantly lower than in the liver of WT mice.

Pttg1 mRNA interference in fibrotic rats also downregulated hepatic expression of the PDGF receptor, Pdgfβ. Specific blockade of the extrahepatic PDGFRβ pathway with adenoviral vectors in CCL4-induced fibrosis rats or systemic PDGF antagonism in bile duct ligated rats led to significantly reduced hepatic fibrosis. Considering that PDGF is the most potent pro-proliferative cytokine for HSCs reduced fibrosis observed after Pttg1 mRNA interference treatment likely is a consequence of blockade of HSC proliferation and inhibition of chemotaxis, which thereby decreases the number of cells able to synthesize ECM proteins. Administration of Pttg1 siRNA to fibrotic rats also affects the regulation of ECM remodelling. In experimental and human cirrhosis, fibrosis appears to be the result of not only excessive ECM synthesis but also reduced degradation. Fibrotic rats receiving C− siRNA for 24, 48 and 72 h. Results are expressed as mean ± SE. *P < 0.05, ***P < 0.001 vs C siRNA. One-way ANOVA with Newman-Keuls post hoc test. B, mRNA expression of Pttg1 and Dlk1 in liver tissue of control (n = 6) and fibrotic rats treated with C siRNA (n = 6) or Pttg1 siRNA (n = 6). *P < 0.05 vs C siRNA; #P < 0.05 vs Pttg1 siRNA. Kruskal-Wallis test with Dunn post hoc test.

**Figure 5** Effect of Pttg1 siRNA on Pttg1 and Dlk1 expression. A, mRNA expression of Pttg1 in CC-1 cells transfected with C siRNA or Pttg1 siRNA for 24, 48 and 72 h.
thereby supporting the concept that PTTG1 activity is a regulator of the ECM degradation pattern in the injured liver mainly by controlling TIMPs activity.

Interestingly, we also observed a tendency towards normalization of most surrogate liver function serum markers. However, this was not the case with transaminases. This finding, previously documented in null Pttg1 fibrotic mice, indicates that Pttg1 mRNA interference has no effect on CCl4-induced hepatotoxicity and further supports the concept that Pttg1 deficiency directly interferes with fibrosis.

Several potential mechanisms mediate the effects of Pttg1 knockdown on hepatic fibrosis. First, the Pttg1 blockade prevents hepatic Dlk1 overexpression. Pttg1 acts as a post-transcriptional regulator of Dlk1, and Dlk1 inhibition results in reduced HSC activation and associated fibrosis. The decrease of Dlk1 expression mediated by Pttg1 downregulation was associated with a significant reduction in activation of HSC, suggesting that PTTG1/DLK1 pathway plays a pivotal role in the hepatic fibroproliferative process. On the other hand, both Pttg1 and Dlk1 share common regulatory mechanisms in which histone deacetylases (HDAC) are involved. Actually, HDAC1 expression has been shown to be responsible for the proliferation of corticotroph cells via Pttg1, whereas HDAC3 activity represses Dlk1 expression in preadipocytes and NIH 3 T3 cells. Second, Pttg1 is involved in the regulation of ECM turnover key proteins. Experimental evidence indicates that Pttg1 is a major regulator of Mmp2 by inducing its secretion and expression. MMP2 plays an important role in remodelling basement membranes as it degrades several components including collagen IV, laminin and fibronectin. The present study shows that Mmp2
expression increases in liver fibrosis, however, Pttg1 interference may have an antifibrogenic effect also by reducing Mmp2 expression and, consequently, by blocking degradation of normal perisinusoidal matrix and promoting activation of quiescent HSC. In this study, we also observed that Pttg1 blocking reduced TIMPs expression. Timp1 and Timp2 are mainly expressed in activated HSC, thus, Timp1 and Timp2 expression could be reduced as a result of diminished HSC activation in these animals. TIMPs also stimulate fibroblast proliferation. Thus, Timp1 and Timp2 downregulation could also contribute to decreased proliferation of activated HSC. A graphical model summarizing the proposed mechanism underlying PTTG1-induced promotion of liver fibrosis is provided in the supplementary information section.

In conclusion, this investigation shows that serial administration of Pttg1 siRNA exerts antifibrotic effects when administered during induction of hepatic damage. Pttg1 gene silencing normalizes expression of Dlk1, arrests activation of HSC, diminishes expression of ECM-related genes and finally decreases hepatic collagen deposition and reduces portal hypertension. Thus, the PTTG1/DLK1 axis may represent a valuable target for the prevention and treatment of liver fibrosis.

CONFLICT OF INTEREST
The authors declare no competing interests. Dr Bruix consults for, advises, and is on the speakers’ bureau and received grants from Bayer-Shering and BTG. He consults for and advises MSD. He consults for and is on the speakers’ bureau for Sirtex. He consults for and received grants from Arqule and Ipsen. He consults for Novartis, Bristol-Myers Squibb, Eisai, Kowa, Terumo, Gilead, Bio Alliance, Roche, AbbVie, Merck, AstraZeneca, Incyte, Quirem, Adaptimmune and Lilly.

ETHICAL APPROVAL
All animal procedures and human samples were approved by the Investigation and Ethics Committee of the Hospital Clinic and Animal Experimentation Committee of the University of Barcelona (Barcelona, Spain). Human samples have consented for research in accordance with ethical guidelines of the 1975 Declaration of Helsinki.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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TABLE 2 Serum markers of liver function in control rats and fibrotic rats treated with C− or Pttg1 siRNAs

|                          | Control (n = 6) | C− siRNA (n = 6) | Pttg1 siRNA (n = 6) |
|--------------------------|----------------|-----------------|--------------------|
| Alanine transaminase (U/L) | 43 ± 6         | 1069 ± 356 ±     | 1226 ± 385 ±       |
| Aspartate transaminase (U/L) | 56 ± 11        | 2342 ± 561 ±     | 924 ± 235 ±        |
| Lactate dehydrogenase (U/L) | 356 ± 27       | 1197 ± 224 ±     | 349 ± 49 ±         |
| Gamma-glutamyl transferase (U/L) | 0.03 ± 0.03  | 5.38 ± 1.68 ±   | 2.40 ± 1.10 ±      |
| Total bilirubin (mg/dL) | 0.00 ± 0.00    | 1.08 ± 0.32 ±    | 0.22 ± 0.12 ±      |
| Total proteins (g/L)  | 49.7 ± 2.2     | 41.0 ± 3.0 ±     | 44.5 ± 1.8 ±       |
| Albumin (g/L)          | 27.6 ± 1.8     | 23.9 ± 1.9       | 27.0 ± 2.1 ±       |
| Total cholesterol (mg/dL) | 55.4 ± 9.9     | 68.29 ± 8.39 ±   | 50.80 ± 6.43 ±     |
| Triglycerides (mg/dL)  | 4.34 ± 1.03    | 57.5 ± 12.7 ±    | 33.0 ± 7.7 ±       |
| Glucose (mg/dL)        | 207 ± 36       | 73 ± 23 ±        | 91 ± 21 ±          |

One-way ANOVA with the Newman–Keuls post hoc test or the Kruskal-Wallis test with the Dunn post hoc test when appropriate. Results are given as mean ± SE.

*P < 0.05; **P < 0.01; ***P < 0.001 vs control.
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