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β-PDGF Receptor Expressed by Hepatic Stellate Cells Regulates Fibrosis in Murine Liver Injury, but Not Carcinogenesis

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Abstract

Background & Aims—Rapid induction of β-PDGF receptor (β-PDGFR) is a core feature of hepatic stellate cell activation, but its cellular impact *in vivo* is not well characterized. We explored the contribution of β-PDGFR-mediated pathway activation to hepatic stellate cell

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The authors disclose no conflicts.

Author contributions
P.K. co-designed research plan, performed experiments, analyzed data, generated figures and drafted manuscript, A.L. and Y.A.L. performed experiments, analyzed data and performed validation of gene array results, A.D. performed flow-cytometric analysis, X.S. performed statistical analysis on data generated through gene array, M.I.F.: performed histological grading, S.T. performed histological grading, C.A. designed and supervised flow-cytometric analysis, P.S. generated original genetic mouse models, designed experiments, interpreted data, Y.H. provided statistical analysis and advice on gene array data, performed bioinformatic analysis and interpretation of data, S.L.F. co-designed research plan, reviewed and edited manuscript

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responses in liver injury, fibrogenesis, and carcinogenesis *in vivo* using genetic models with divergent β-PDGFR activity, and assessed its prognostic implications in human cirrhosis.

Methods—The impact of either loss or constitutive activation of β-PDGFR in stellate cells on fibrosis was assessed following carbon tetrachloride (CCl_4) or bile duct ligation. Hepatocarcinogenesis in fibrotic liver was tracked after a single dose of diethylnitrosamine (DEN) followed by repeated injections of CCl_4. Genome-wide expression profiling was performed from isolated stellate cells that expressed or lacked β-PDGFR to determine deregulated pathways and evaluate their association with prognostic gene signatures in human cirrhosis.

Results—Depletion of β-PDGFR in hepatic stellate cells decreased injury and fibrosis *in vivo*, while its auto-activation accelerated fibrosis. However, there was no difference in development of DEN-induced pre-neoplastic foci. Genomic profiling revealed ERK, AKT, and NF-kB pathways and a subset of a previously identified 186-gene prognostic signature in hepatitis C virus (HCV)-related cirrhosis as downstream of β-PDGFR in stellate cells. In the human cohort, the β-PDGFR signature was not associated with HCC development, but was significantly associated with a poorer outcome in HCV cirrhosis.

Conclusions—β-PDGFR is a key mediator of hepatic injury and fibrogenesis *in vivo* and contributes to the poor prognosis of human cirrhosis, but not by increasing HCC development.

Keywords

HCC; cirrhosis; receptor tyrosine kinase; gene expression signatures; pathway analysis

Introduction

Among mitogenic pathways in stellate cells, signaling by the beta platelet-derived growth factor receptor (β-PDGFR) is the most potent [1, 2]. Expression of PDGF receptors is low in healthy liver, but dramatically increases in stellate cells during injury [2, 3]. In both mice and humans, the PDGF signaling network is comprised of four ligands, PDGF A–D, which transduce their signals through dimeric transmembrane receptors α- and β-PDGFR, which can form hetero- and homodimers [4]. Upon ligand binding, receptor dimerization provokes phosphorylation of the tyrosine residues within the intracellular domain, leading to activation of the Fas-MAPK pathway, signaling through the PI3K-AKT/PKB pathway and activation of PKC family members [5].

Antagonism of β-PDGFR has been an appealing target to treat hepatic fibrosis. Indeed, our previous study and those of others [6–8] have demonstrated that the RTK inhibitor imatinib mesylate (Gleevec®) whose targets include β-PDGFR, inhibits stellate cell activation and reduces fibrosis.

Recent evidence links the behavior of stromal cells, especially driven by the ligand PDGF-B, not only to the pathogenesis of fibrosis, but also to inflammation, regeneration and cancer [9]. Sorafenib (Nexavar®), a multi-receptor tyrosine kinase inhibitor whose targets include β-PDGFR, remains the only drug approved for treatment of advanced, non-resectable HCC [10].

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Despite the suggestion that promotion of fibrosis by β-PDGFR can accelerate HCC development, this important question has not been addressed experimentally. Here we have specifically explored the contribution of β-PDGFR signaling by activated hepatic stellate cells to injury, fibrosis and cancer by exploiting complementary genetic models using a Cre-Lox strategy, one in which the receptor was deleted in stellate cells, or another in which it was auto-activated. Importantly, we have examined whether β-PDGFR signaling in hepatic stellate cells is linked in human cirrhosis to overall prognosis.

Methods

For detailed description of methods see Supplementary Methods.

Animals

β-PDGFR$^{fl/fl}$ mice, as previously described [11], (on the 129S4/SvJaeSor background) were crossed with a transgenic FVB line expressing Cre-recombinase under control of the human glial fibrillary acidic protein (GFAP) promoter to generate β-PDGFR$^{fl/fl}$ GFAP-Cre mice with a deletion of β-PDGFR in stellate cells – this GFAP promoter has been successfully validated in prior studies as active in hepatic stellate cells [12, 13]. To create animals with constitutively activated β-PDGFR in stellate cells, β-PDGFR$^{betaJ/+}$ mice, as previously described [14], (on the 129S4/B6 background) were also crossed with a transgenic GFAP-Cre line to generate β-PDGFR$^{betaJ/+}$ GFAP-Cre mice. These animals harbor hepatic stellate cells with autoactivation of β-PDGFR, owing to an activating mutation knocked into the β-PDGFR locus, plus addition of a lox-stop-lox cassette between the splice acceptor and the initiating codon of the cDNA [14].

Models of Murine Liver injury and Fibrosis

Liver fibrosis was induced either by ligation of the common bile duct (BDL) [15] or by intraperitoneal (i.p.) injections of carbon tetrachloride (CCl_4, Sigma, St. Louis, MO) [16]. For acute CCl_4 injury studies, mice received a total of 3 i.p. injections (alternating days) of either corn oil or 10% CCl_4 (diluted in corn oil) at a dose of 0.5 μl/g body weight. For the chronic injury model, mice received i.p. injections of CCl_4 3 times per week for a total of 6 weeks.

Induction of Carcinogenesis

Mice received a single dose of diethylnitrosamine (DEN, Sigma, St. Louis, MO) (25μg/g bw i.p.) at day 15 post-partum. Starting two weeks after DEN, mice received a total of 22 injections of CCl_4 (0.5μl/g bw i.p., 1 injection/week) [17]. Mice were sacrificed 48 hours following the last CCl_4 injection. Nodule number and size was documented as described by counting and measuring the diameter of each lesion using a caliper.

Primary Hepatic Stellate cell Isolation and Cell Culture

Mouse hepatic stellate cells were isolated from β-PDGFR$^{fl/fl}$ GFAP-Cre negative and β-PDGFR$^{fl/fl}$ GFAP-Cre positive mice by enzymatic pronase and collagenase digestion and density gradient centrifugation as previously described [18]. Cells were cultured with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. Cells

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were either treated with or without PDGF-B [10 ng/ml] (Peprotech, Princeton, NJ) diluted in albumin (vehicle) containing serum-free media (DMEM).

Histologic and Immunohistochemical Studies

Liver samples were formalin-fixed, paraffin-embedded, sectioned at 4 μm, and processed routinely for H&E staining. Sirius Red, combined with morphometry, was used to quantify collagen using Bioquant image analysis software (Bioquant Image Analysis Corporation, Nashville, TN). Immunohistochemical staining of αSMA and desmin was performed on formalin-fixed, paraffin-embedded liver sections with a rabbit polyclonal antibody (Abcam, Cambridge, England). A pathologist blindly scored 5 random areas per slide for necrosis, inflammation and dysplasia.

Genome-wide expression profiling

Genome-wide gene expression profiling of mouse primary hepatic stellate cells was performed, in triplicate, by using MouseWG-6 v2.0 Expression BeadChip (Illumina) according to the manufacturer's protocol. Raw scanned data were normalized by using cubic spine algorithm implemented in the GenePattern genomic analysis toolkit (www.broadinstitute.org/genepattern) [19]. Probe-level expression data were collapsed into gene-level by calculating the median of multiple probes, and converted to human genes based on an orthologous mapping table provided by the Jackson laboratory (www.informatics.jax.org). The dataset (GSE#52253) is available at NCBI Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo).

Bioinformatics and Statistical Analysis

Enrichment of molecular pathways was evaluated by Gene Set Enrichment Analysis (GSEA) [20] on a comprehensive gene set collection in Molecular Signatures Database (see Supplementary Methods).

Results

β-PDGFR Expression is Induced Upon Liver Injury *In Vivo* and *In Vitro*

We generated a mouse line in which the expression of β-PDGFR was deleted in hepatic stellate cells by crossing β-PDGFR$^{fl/fl}$ mice with animals expressing Cre-recombinase under the human glial fibrillary acidic protein promoter (GFAP-Cre) (Suppl. Fig. 1A) [13, 21].

To first confirm the induction of β-PDGFR following acute injury, β-PDGFR$^{fl/fl}$ GFAP-Cre negative animals were treated with CCl_4 in three doses in one week. Whole liver lysates contained increased β-PDGFR expression and phosphorylation, as well as up-regulation of αSMA (Fig. 1A).

We next analyzed isolated hepatic stellate cells from β-PDGFR$^{fl/fl}$ Cre negative mice (β-PDGFR) as well as their β-PDGFR$^{fl/fl}$ Cre expressing littermates (β-PDGFR) (Fig. 1B, C). Isolated stellate cells were maintained in primary culture for six days following isolation. To first validate the knock down and diminished activation of β-PDGFR in the Cre expressing population, cell lysates were analyzed via immunoblot after incubation with either vehicle or

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PDGF-BB (Fig. 1B). Stellate cells from β-PDGFR mice displayed attenuated receptor expression and lack of activation at baseline and after 30 minutes of ligand exposure. Next, the expression of collagen I and αSMA were compared in primary stellate cells of the two groups. Stellate cells isolated and cultured for 6 days displayed significant knock down of the receptor in the β-PDGFR group, with substantially reduced expression levels of collagen I and αSMA compared to stellate cells from β-PDGFR animals (Fig. 1C). β-PDGFR expression was upregulated during hepatic injury and correlates with stellate cell activation *in vitro*.

Deletion of β-PDGFR in Stellate Cells Attenuates Liver Fibrosis *In Vivo*

Since mice responded to acute liver injury with up-regulation of β-PDGFR on stellate cells associated with fibrogenic markers, we analyzed control and β-PDGFR animals (Suppl. Fig. 1A) following acute (1 week) and chronic (6 weeks) liver injury. To do so, we injected both groups with either oil or CCl_4 (3 i.p. injections weekly).

Macroscopically, there were no differences in the livers between control and β-PDGFR littermates after treatment (Suppl. Fig. 1B, C), but less necrosis was present at the early time point (Suppl. Fig. 2A, B). Although there was a trend in reduced inflammation in the livers of β-PDGFR mice after one week of injections (Suppl. Fig. 2B), comprehensive flow cytometry did not confirm any differences in the numbers or subsets of lymphocytes at baseline or after injury between wild type and β-PDGFR littermates (data not shown). β-PDGFR-deficiency led to significantly decreased collagen deposition after chronic hepatic injury as assessed by Sirius Red morphometry and hydroxyproline assay (Fig. 2 A, B, C). Serum transaminase levels (AST and ALT) were increased, especially after 6 weeks (Fig. 2D). As expected, livers with β-PDGFR deletion in stellate cells expressed less α*SMA* and significantly less *collagen* α*1(I)* mRNAs (Fig. 2E). In order to validate the effect of diminished β-PDGFR expression on the expansion of activated HSCs following CCl_4 treatment, we quantified the αSMA-positive tissue area in both groups (Suppl. Fig. 3A). Both after a short-term, and more strikingly, after long-term injury, mice of the β-PDGFR group had reduced expansion of activated HSCs versus controls. This finding was further validated using whole liver lysates of livers after 6 weeks of CCl_4 treatment (Suppl. Fig. 3B). Here, the decreased expression of β-PDGFR resulted in a diminished expression of αSMA. As an additional marker for HSCs and to measure the expansion of HSCs during injury within the liver, desmin staining was performed on tissue sections following 1 week of CCl_4 treatment (Suppl. Fig. 4). In these mice, there was a significant decrease in desmin-positive tissue quantification in β-PDGFR mice versus controls.

Similar but less striking results were apparent in the bile duct ligation model (see Suppl. Fig. 5).

Loss of β-PDGFR in Stellate Cells Diminishes Their Proliferative Response *In Vivo*

To determine whether increased fibrosis upon hepatic injury was due to β-PDGFR mediated stellate cell proliferation, we performed *in vivo* labeling of dividing stellate cells. Mice of both groups received a total of three i.p. injections of CCl_4 every 48 hours; 44 hours after the last injection, mice received a single i.p. injection of BrdU (1.5mg/150μl PBS), and cells

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were isolated four hours thereafter. Isolated cells were stained for CD45 and BrdU and then analyzed by flow cytometry. UV autofluorescence was used to distinguish stellate cells from non-fluorescent cells, while the expression of CD45 was used to discriminate $CD45^{-}$ stellate cells from other non-parenchymal cells (Suppl. Fig. 6A). Based on this analysis, 44.3% of stellate cells from β-PDGFR mice had incorporated nuclear BrdU compared to 16.3% in β-PDGFR cells (Suppl. Fig. 6B), indicating that β-PDGFR expression of stellate cells correlates with significantly increased proliferation during hepatic injury.

Constitutively Activated β-PDGFR in Stellate Cells Amplifies Liver Fibrosis *In Vivo*

To confirm the vital role of β-PDGFR during hepatic fibrosis, we analyzed a mouse line in which the expression of a β-PDGFR auto-activating mutant was induced in hepatic stellate cells by crossing β–$PDGFR^{betaJ/+}$ mice with GFAP-Cre transgenic animals (Suppl. Fig. 7A). We compared $PDGFR^{betaJ/+}$ Cre negative (β-PDGFR) and their $PDGFR^{betaJ/+}$ Cre positive (βJ) littermates following three weekly i.p. injections of either corn oil or CCl_4 (0.5 μl/g body weight) over one (acute) or six (chronic) weeks (Fig. 3). The auto-activating βJ group displayed differences in fibrotic tissue area versus control animals upon oil injections (Fig. 3A, B, C). Differences were also significant following 6 weeks of CCl_4, with βJ mice displaying significantly increased collagen deposition (Fig. 3A, B, C). Consistent with these findings, mutant βJ expression on stellate cells led to significantly more *collagen* α*1(I)* and α*SMA* mRNA expression upon CCl_4 treatment (Fig. 3D).

There were no macroscopic differences between control and mutant βJ littermates before or after treatment (Suppl. Fig. 7B) but there was an increase in liver to body weight ratio in both treatment groups after 1 and 6 weeks (Suppl. Fig. 7C). Serum transaminase levels increased proportionate to treatment duration, without differences between β-PDGFR and βJ littermates (Suppl. Fig. 7D). Both groups displayed increased inflammation and necrosis (Suppl. Fig. 8A, B).

We quantified the desmin-positive area, as a reflection of stellate cell expansion (Fig. 3E). Using this approach, mice with auto-activating β-PDGFR displayed increased desmin-positive tissue area upon acute injury (Fig. 3F). The auto-activating mutation of β-PDGFR led to increased stellate cell proliferation and hepatic fibrosis upon acute and chronic injury.

β-PDGFR Deficiency in Stellate Cells has no Impact on the Carcinogenic Response

Since the majority of HCCs develop within cirrhotic livers, we assessed the contribution of β-PDGFR signaling in stellate cells to the development of dysplastic nodules following DEN plus CCl_4 (Suppl. Fig. 9A) [17]. This regimen reportedly mimics the permissive environment from which regenerative nodules and ultimately HCCs arise [12, 17].

After long-term treatment with both a carcinogen as well as CCl_4, β-PDGFR accumulated less collagen as assessed by Sirius Red morphometry (Fig. 4A, B), associated with reduced *collagen* α*1(I)* mRNA (Suppl. Fig. 9G). However, loss of β-PDGFR had no impact on the total number of lesions, the maximal nodule size, or the liver weight to body weight ratio (Suppl. Fig. 9B, C, D, E). Of note, careful expert analysis revealed the lesions to be dysplastic nodules, not true HCCs. Histologically, liver sections showed a similar

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appearance of dysplastic nodules between both β-PDGFR and β-PDGFR mice as assessed by a blinded pathologist (Suppl. Fig. 9F).

These data indicate that the lack of β-PDGFR expression by stellate cells attenuates fibrosis progression in chronic injury from CCl_4, but does not protect against the development of dysplastic nodules when combined with a carcinogen.

Constitutively Activation of β-PDGFR in Stellate Cells does not lead to an Increased Carcinogenic Response

To further reflect these findings, we used βJ mice and their control littermates to assess their response towards the combined treatment with DEN and CCl_4 (Suppl. Fig. 10). Macroscopically, there was no difference in overall quantification of dysplastic nodule formation (Suppl. Fig. 10), regarding tumor number, the largest tumor diameter and overall liver weight to body weight ratio (Suppl. Fig. 10B, C, D, E). Histologically, the grade of dysplasia was not different among βJ and control mice (Suppl. Fig. 10F). Overall, the activation level of β-PDGFR did not correlate with formation of dysplastic nodules in this murine model.

Prognostic Relevance of β-PDGFR-mediated Signaling in Human Cirrhosis

We previously identified and validated a 186-gene signature in liver that predicts prognosis of patients with liver cirrhosis [22, 23]. The signature is assumed to reflect signals of molecular deregulation from multiple cell types in the cirrhotic tissue microenvironment that drive disease progression. We hypothesized that activation of β-PDGFR signaling in stellate cells contributes at least a part of the prognostic gene signature. Using this dataset, we examined whether the stellate cell-derived β-PDGFR or β-PDGFR gene signature was present in the human cirrhosis cohort and determined its prognostic association. To do so, we performed Gene set enrichment analysis (GSEA) to evaluate induction of the 186-gene signature in isolated stellate cells from our β-PDGFR-wild-type and knockout mice. Of note, we observed a statistically significant enrichment of poor-prognosis- and good-prognosis-correlated signature genes in the wild-type and knockout stellate cells, respectively (false discovery rate <0.25) (Fig. 4C a, b). Genes up-regulated in the β-PDGFR knockout cells (i.e., genes suppressed by β-PDGFR pathway activation) (Supplementary Table 2), were associated with a good prognosis (Fig. 4C a). Likewise, genes up-regulated by β-PDGFR activation showed an association with a high overall risk of mortality in HCV cirrhotic patients (Fig. 4C b), suggesting that the pathway activation itself is partially linked to the risk of liver disease and fibrosis progression. Genes involved in inflammation and cell survival such as NFKB2, IER3, IFI30, and BCL2 contributed to the enrichment within the poor prognosis signature (Table 1). However, genes previously implicated in hepatocarcinogenesis such as EGF [24] were not involved in the enrichment, suggesting that β-PDGFR signaling in stellate cells is not directly involved in the process of hepatocarcinogenesis. We verified the expression levels of the top five differentially regulated genes of β-PDGFR-wild-type and knockout mice as selected by DNA microarray using quantitative real-time PCR (Fig. 4D). These data provide evidence for a strong correlation between microarray data as well as PCR from hepatic stellate cells isolated from mice of both groups. For Ingenuity Pathway Analysis of the 186-gene signature within

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hepatic stellate cells of β-PDGFR control compared to knockout mice, see Suppl. Table 2. Collectively, these results suggest that activation of β-PDGFR signaling in stellate cells has downstream consequences that drive disease progression and poor prognosis of human cirrhosis, but not through increased risk of HCC development.

Discussion

In the current study, we established two divergent genetic mouse models, by using a knockout or auto-activation of β-PDGFR, to assess the impact of titrating β-PDGFR expression on stellate cell and its contribution to liver injury and fibrosis. Deleting β-PDGFR on hepatic stellate cells impairs their fibrogenic potential *in vivo*, leading to decreased expression of αSMA and collagen α1(I), and reducing their proliferation upon injury. Although previous studies have not directly linked β-PDGFR to collagen expression, here we demonstrate that the lack of β-PDGFR on primary hepatic stellate cells leads to decreased expression of collagen I after treatment with PDGF-B. Our data establish that β-PDGFR activation increases fibrosis accumulation at least in part through increased stellate cell numbers based on BrdU incorporation in stellate cells *in vivo* and subsequent FACS analysis. We also emphasize that while specificity of human GFAP-Cre expression has been questioned [25], both this study and separate reports [12, 13] underscore its utility to drive stellate cell cre expression. Moreover, it has been shown that β-PDGFR is only expressed in activated hepatic stellate cells and some vascular endothelial cells in liver thereby minimizing potential hepatic off-target effects of the GFAP-Cre expression since only stellate cells will be affected by deletion of β-PDGFR. One major issue is that differences in the recombination of different floxed alleles or Cre reporters cannot be ruled out completely.

Our data begin to define nuanced and very specific links between some pathways of stellate cell activation, and prognosis of cirrhosis or incidence of HCC, but not others. For example, recent studies strongly implicate EGFR signaling and clinical outcomes in human HCC [24, 26, 27], yet in contrast to a previous report [28] our findings do not establish a similar link for β-PDGFR signaling, despite its clear contribution to fibrosis. EGF was not among the 14 genes that lead to an enrichment of the phenotype for poor prognosis in GSEA downstream of β-PDGFR signaling (Table 1, Suppl. Table 2). Although sorafenib, which is the only approved molecular therapy for HCC, targets multiple receptor tyrosine kinases, including β-PDGFR, our data suggest that the antineoplastic benefit of sorafenib may not be due to its impact on β-PDGFR signaling alone, but rather on other RTK pathways, especially Ras/Raf kinase, HGF and VEGF signaling [29]. We also emphasize that expert histologic assessment failed to confirm the presence of true HCCs but rather only preneoplastic, dysplastic nodules, undermining the potential value of this model in generating true HCCs, in contrast to what has been previously proposed [28].

β-PDGFR activity in stellate cells clearly contributes to progressive disease during chronic liver injury, consistent with the finding that the β-PDGFR gene signature in primary hepatic stellate cells correlates with poor overall outcome in our human cohort. However, the β-PDGFR-related difference in human outcomes is not due to enhanced tumor development, consistent with the findings in our murine model. Thus, β-PDGFR signaling may contribute to poor outcomes in HCV cirrhosis through its impact on fibrosis but not on carcinogenesis.

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These findings indicate that while β-PDGFR is an attractive anti-fibrotic target, it may not be a suitable direct target to inhibit the development of neoplasia in cirrhotic liver, in contrast to other RTK pathways, especially EGFR signaling [30], where prophylactic receptor antagonism is a viable strategy for reducing the risk of cancer more directly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

β-PDGFR	beta platelet-derived growth factor receptor
HCV	hepatitis C virus
CCl_4	carbon tetrachloride
DEN	diethyl nitrosamine
GFAP	glial fibrillary acidic protein
BDL	bile duct ligation
bw	body weight
AST	aspartate aminotransferase
ALT	alanine aminotransferase
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
αSMA	alpha smooth muscle actin
H&E	hematoxylin and eosin
BrdU	5-bromo-2'-deoxyuridine
CD45	cluster of differentiation 45
UV	ultraviolet
PBS	phosphate buffered saline

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HCC	hepatocellular carcinoma
IL1R	interleukin 1 receptor
GSEA	gene set enrichment analysis
FACS	fluorescence-activated cell sorting
VEGF	vascular endothelial growth factor
HGF	hepatocyte growth factor
RTK	receptor tyrosine kinase
EGFR	epidermal growth factor receptor
NES	normalized enrichment score
FDR	false discovery rate

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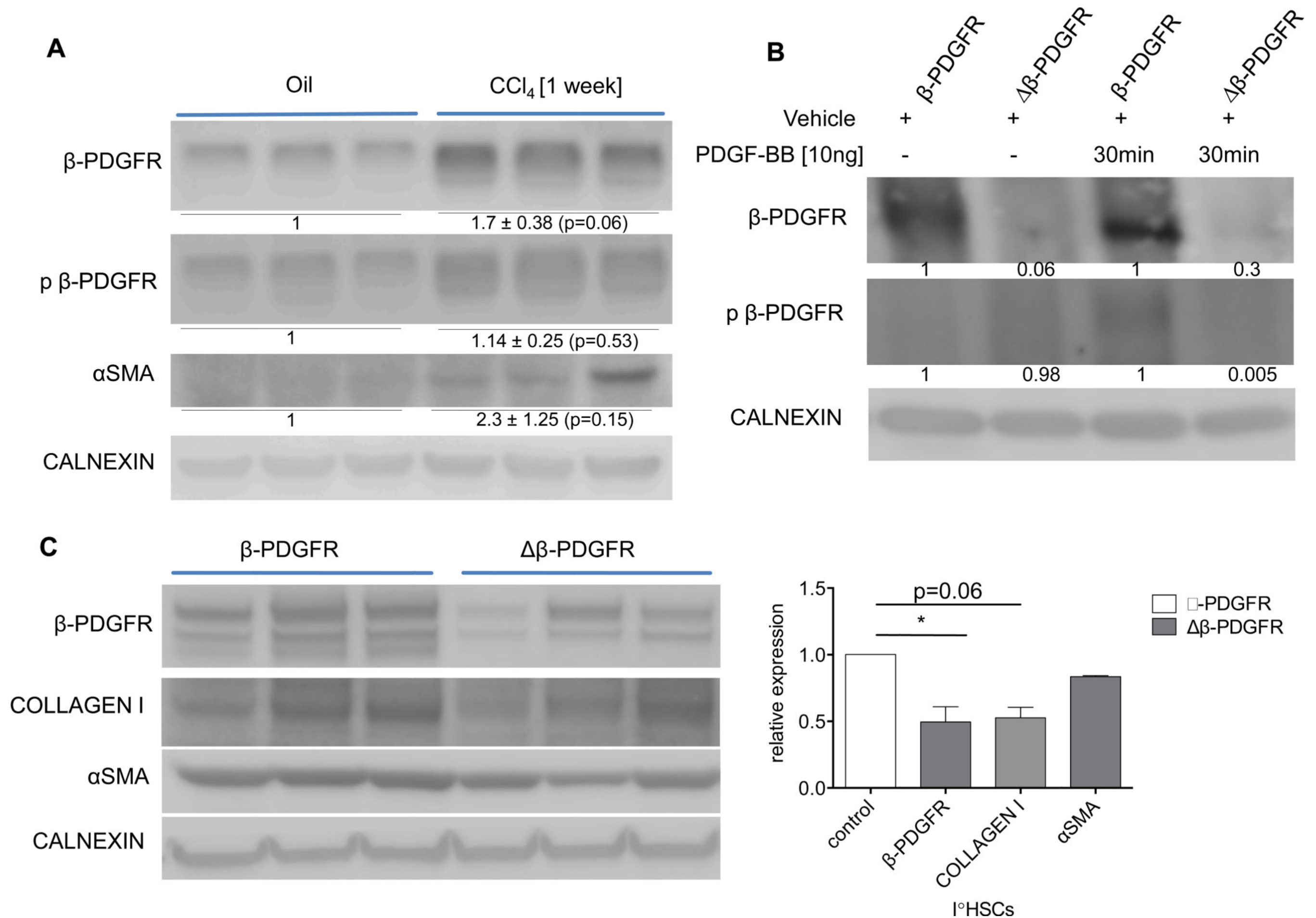

Fig. 1. β-PDGFR expression correlates with activation of mouse hepatic stellate cells *in vivo* and *in vitro*

(A) Mice were injected three times with either oil or CCl_4 to induce acute liver injury and were sacrificed two days after the last injection. Immunoblot of whole liver lysates demonstrating increased expression of β-PDGFR, phospho-β-PDGFR and αSMA upon injury.

(B, C) β-PDGFR mice and control littermates were injected once with CCl_4 followed by isolation of HSCs 48 hours thereafter. Primary HSCs were kept in culture for 6 days.

(B) Immunoblot demonstrates phosphorylation of the receptor in control animals upon ligand exposure and lack of receptor activation in HSCs of β-PDGFR animals.

(C) Immunoblot showing decreased HSC activation of β-PDGFR mice in culture compared to wild type β-PDGFR mice, with reduced expression of Collagen I and αSMA. Graph indicating densitometric analysis of each band, verifying significant knock down of β-PDGFR in primary HSCs of β-PDGFR mice and decreased expression of HSC activation markers.

Data represent the mean value of at least 3 separate experiments (*$p<0.05$, error bars indicate SEM). 3 animals per condition were used in each experiment. Protein ratios (normalized to calnexin) were used to quantify the fold change relative to control, and are shown below each blot.

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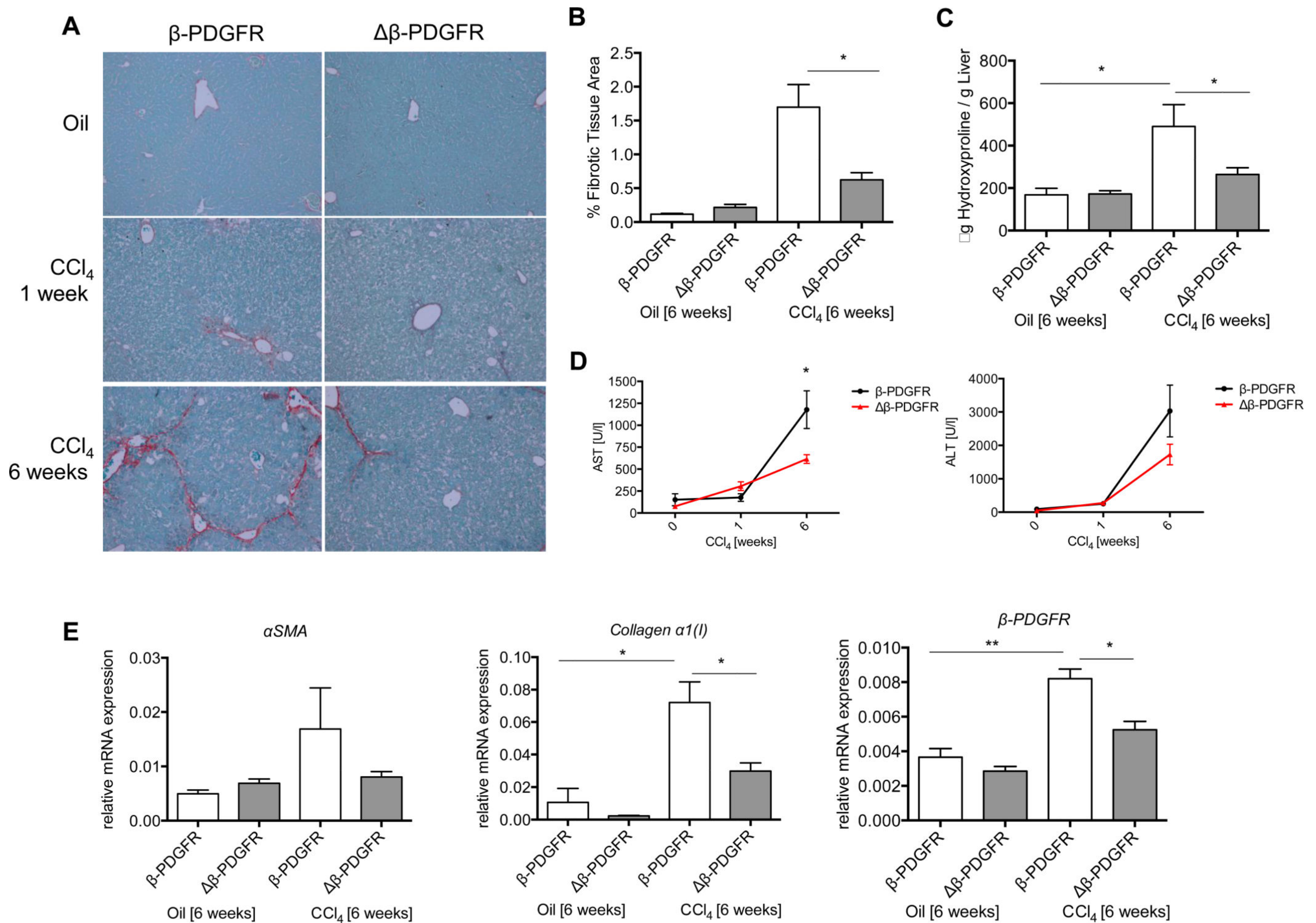

Fig. 2. Loss of β-PDGFR on HSCs leads to decreased collagen deposition *in vivo*

(A–E) β-PDGFR and control mice were injected with CCl_4 over either one or six weeks to induce acute or chronic liver injury.

(A) Sirius Red staining of paraffin embedded liver sections following acute or chronic liver injury depicts significantly lower collagen deposition after chronic injury (magnification 200×).

(B) Graph displays the percentage of liver area positive for Sirius Red staining measured by morphometric analysis. The area of fibrotic tissue is significantly reduced within β-PDGFR animals compared to controls.

(C) Measurement of hydroxyproline content per gram of whole liver after 6 weeks of CCl_4 reflects reduced hydroxyproline content in livers of β-PDGFR versus controls.

(D) Levels of serum AST and ALT during acute and chronic injury.

(E) Whole liver mRNA expression of *Collagen α1(I)*, α*SMA*, β-*PDGFR* after 6 weeks of CCl_4 treatment confirm increased expression of stellate cell activation genes within control animals upon injury, as well as lack of increase within the β-PDGFR group.

All figures represent the mean of at least n=3 animals per experimental group. mRNA is expressed normalized to *Gapdh* (*p < 0.05, **p< 0.001; error bars indicate SEM).

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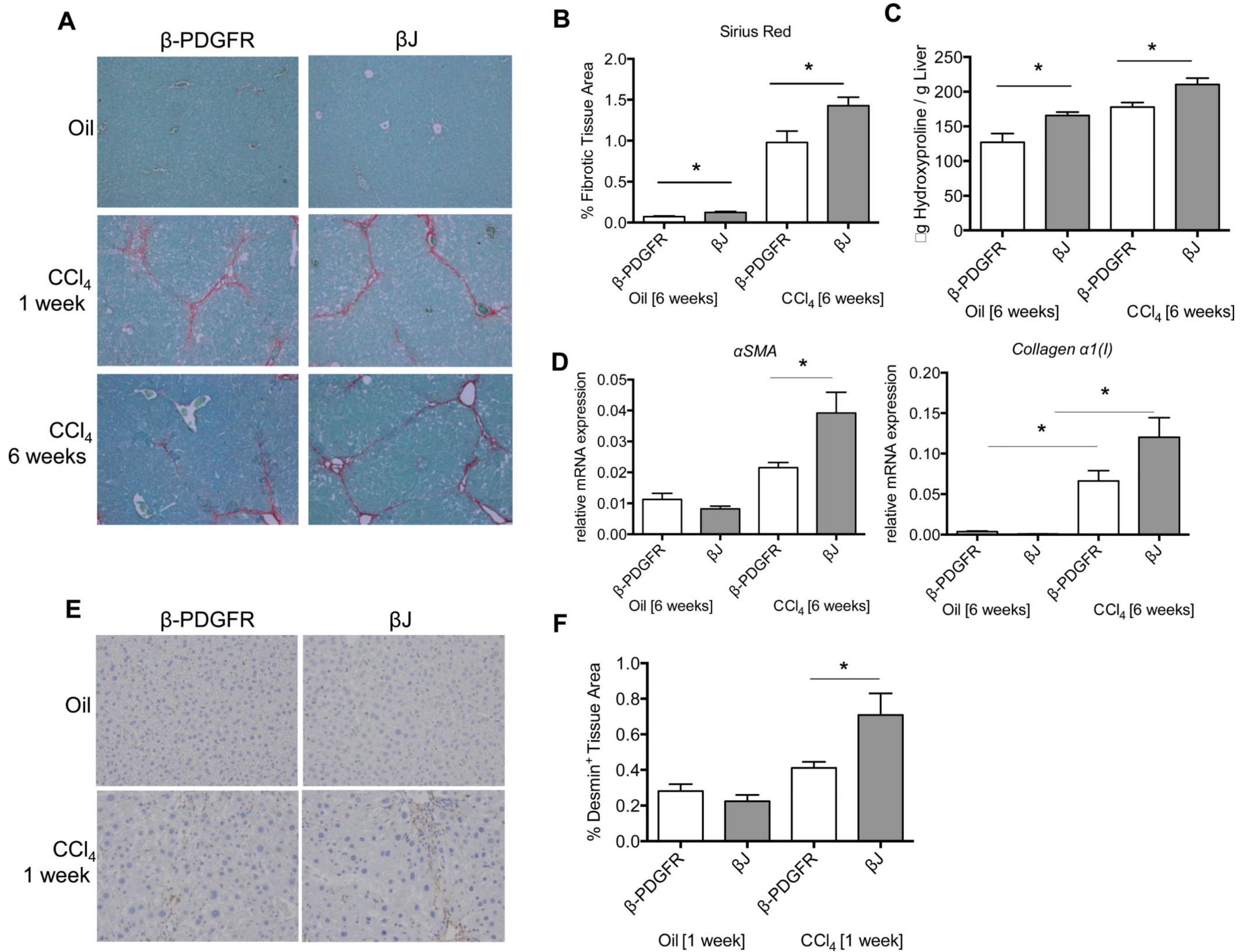

Fig. 3. The 'βJ' constitutively activating mutant of β-PDGFR on stellate cells leads to increased collagen deposition upon injury *in vivo*

(A–D) βJ and control mice were injected with CCl_4 over either one or six weeks to induce acute or chronic liver injury.

(A) Sirius Red staining of paraffin embedded liver sections following acute or chronic liver injury depicts significantly higher collagen deposition after acute and chronic injury (magnification 200×).

(B) Graph shows the percentage of liver area positive for Sirius Red staining measured by morphometry. The area of fibrotic tissue is significantly increased in livers of βJ animals compared to controls.

(C) Increased hydroxyproline content per gram of whole liver after 6 weeks of CCl_4 in livers of βJ mice compared to controls.

(D) Whole liver mRNA expression of α*SMA* and *Collagen* α*1(I)* after 6 weeks of CCl_4 treatment confirms increased expression of stellate cell activation genes in βJ animals upon liver injury.

(E) Desmin staining of paraffin embedded liver sections following acute liver injury depicts increased stellate cell expansion within livers of βJ mice versus controls.

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(F) Graph shows percentage of tissue area positive for desmin measured by morphometry. All figures represent the mean of at least n=5 animals per experimental group. mRNA is expressed normalized to *Gapdh* (*$p < 0.05$, **$p < 0.001$; error bars indicate SEM).

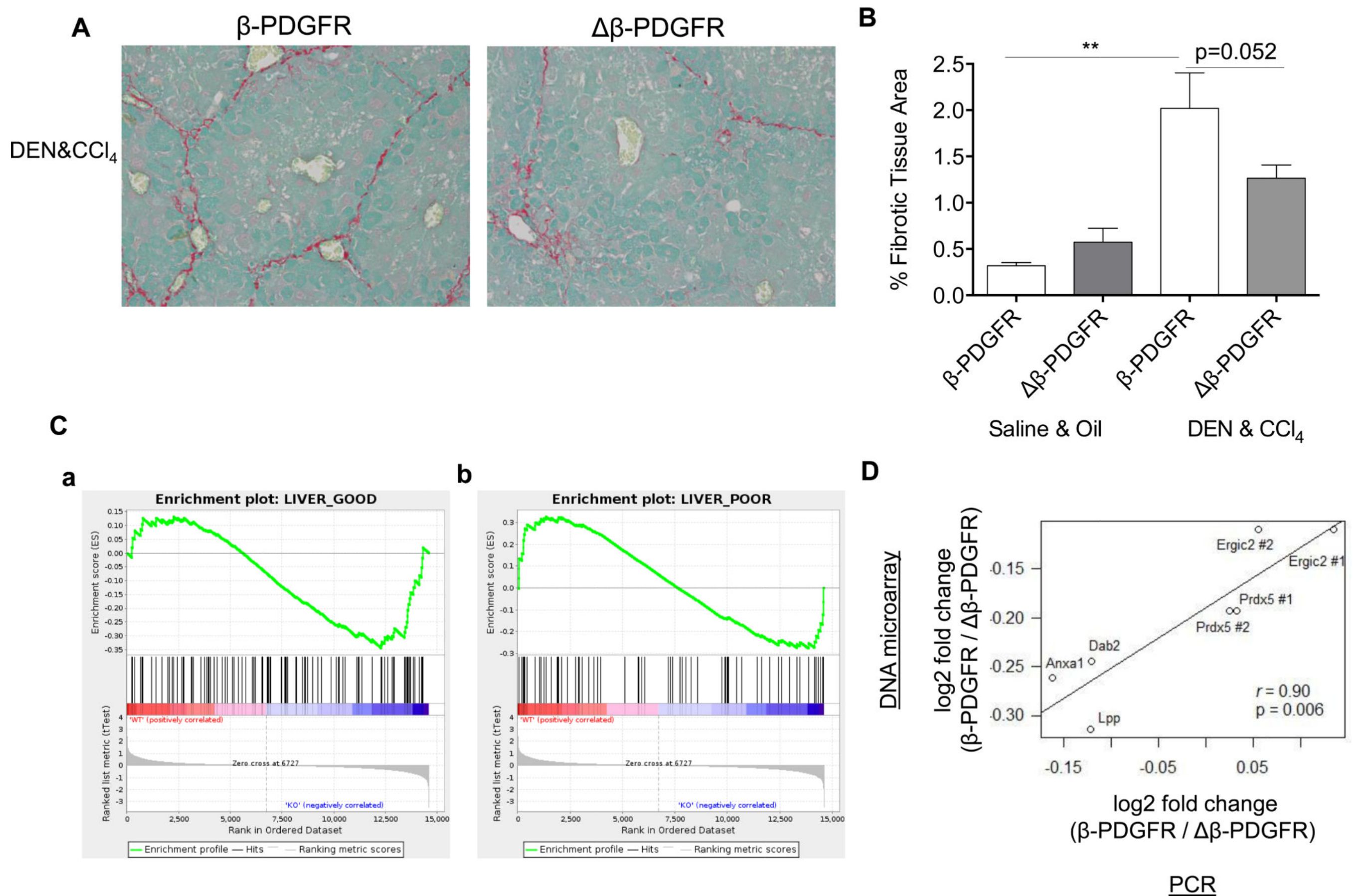

Fig. 4. Deletion of β-PDGFR reduces fibrosis but not tumor burden in mice treated with DEN and chronic CCl_4, and is linked to better outcome in patients with cirrhosis

Mice were treated with a single dose of DEN at day 15, followed by weekly injections with CCl_4 and sacrificed 48h after the last of 22 injections with CCl_4.

(A) Paraffin embedded liver sections were stained with Sirius Red to assess fibrotic tissue content (magnification 200×).

(B) Decreased collagen area measured by morphometry in livers of β-PDGFR mice. The bar graph represents the mean of n=5 animals per experimental group. (**p< 0.001; error bars indicate SEM).

(C) GSEA plots demonstrate β-PDGFR-dependent association with gene signatures for either a good or poor prognosis in an HCV cirrhotic patient cohort.

Gene array samples of primary hepatic stellate cells isolated from either β-PDGFRfl/fl GFAP-Cre negative (indicated as WT) or β-PDGFRfl/fl GFAP-Cre positive (indicated as KO) mice were correlated with gene signatures for good or poor overall prognosis of a human HCV cirrhosis cohort.

(a) Association of the β-PGDFR-knockout gene signature with a good outcome in liver cirrhosis. Enrichment of the β-PGDFR-knockout gene signature was evaluated in association with the risk of overall mortality in 216 HCV-related cirrhosis patients with early-stage cirrhosis (n=216). NES=−1.14, nominal p=0.21, FDR=0.22.

(b) Association of the β-PGDFR gene signature with a poor outcome in HCV cirrhosis. Enrichment of the β-PGDFR gene signature was evaluated in association with the risk of

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overall mortality in 216 HCV-related cirrhosis patients with early-stage cirrhosis (n=216). NES=1.11, nominal p=0.25, FDR=0.23.

Genes were evaluated using GSEA.

(D) Comparison of β-PDGFR knockout-mediated differential gene expression between DNA microarray and RT-qPCR was performed choosing five of the top differentially expressed genes (Anxa1, Dab2, Ergic2, Lpp, and Prdx5) between wild type and β-PDGFR-knockout mice as selected from DNA microarray data. To verify the differential expression in RT-qPCR, 7 pairs of primers (2 pairs for Ergic and Prdx5) were designed and the same RNA aliquots were assayed in triplicate.

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Table 1

Induction of 186-gene human prognostic gene signature in primary stellate cells from wild-type and β-Pdgfr knockout mice.

a) Poor-prognosis-associated genes induced in wild-type mouse stellate cells

Human gene symbol	Mouse gene symbol	t-statistic
DAB2	Dab2	1,35
LPP	Lpp	1,28
ANXA1	Anxa1	1,28
NFKB2	Nfkb2	0,96
EPM2AIP1	Epm2aip1	0,90
SERPINB2	Serpinb2	0,90
ITGA9	Itga9	0,83
IQGAP1	Iqgap1	0,81
IER3	Ier3	0,74
CCDC6	Ccdc6	0,54
COL4A1	Col4a1	0,53
IFI30	Ifi30	0,46
ANXA3	Anxa3	0,44
BCL2	Bcl2	0,40
SERPINB8	Serpinb8	0,37

b) Good-prognosis-associated genes induced in Pdgfrb-knockout mouse stellate cells

Human gene symbol	Mouse gene symbol	t-statistic
DAD1	Dad1	−0,30
VPS41	Vps41	−0,30
ERCC5	Ercc5	−0,32
RRM1	Rrm1	−0,32
ACOT2	Acot2	−0,35
GCGR	Gcgr	−0,38
RFC2	Rfc2	−0,40
TIMM8A	Timm8a1	−0,40
TXN2	Txn2	−0,51
GGCX	Ggcx	−0,53
ZNF185	Zfp185	−0,54
HSPE1	Hspe1	−0,55
FAM129A	Fam129a	−0,56
F9	F9	−0,58
PSMB3	Psmb3	−0,60
MSH6	Msh6	−0,61
XPA	Xpa	−0,67
ZER1	Zer1	−0,69
GHR	Ghr	−0,80

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b) Good-prognosis-associated genes induced in Pdgfrb-knockout mouse stellate cells

Human gene symbol	Mouse gene symbol	t-statistic
ATP5D	Atp5d	−0,88
PMM1	Pmm1	−0,90
NENF	Nenf	−0,94

t-statistic indicates degree of differential expression between wild-type and Pdgfrb-knockout mouse stellate cells. Positive values indicate up-regulation in wild-type and negative values indicate up-regulation in knockout.