Supplemental Material and Methods

1. Generation of Smad7 transgenic mice

Transgenic mice were generated on FVB/NHSD genetic background with isolated and purified Smad7-expression vector DNA. Offspring were analysed for presence of the transgene by transgene-specific PCR. Preliminary to the experiments presented in this manuscript, Smad7 transgenic mice were backcrossed onto C57Bl/6J genetic background. After breeding with Alb-Cre transgenic mice, Smad7 transgenic mice delivered double-transgenic offspring in expected ratios.
Animals were housed in a constant day–night cycle of 12 h and fed a standard chow diet with water access ad libitum. Animal experiments were performed in accordance with the European Council Directive of November 24, 1986 (86/609/EEC) and were approved by the local Authorities.

Smad7-Expression vector

Four identical 800bp insulator elements of the human β-globin cluster [1,2] were cloned into pBR322*3 vector [3]. The albumin enhancer/promoter region [4,5] was ligated into an expression vector containing a rabbit β-globin intron and hGH polyA site [6]. The fragment comprising promoter, intron and polyA site was purified and inserted between two pairs of insulators into the previously generated pBR322*3 derivative. A lacZ gene with a nuclear localisation signal followed by a hGH polyA site and flanked by two loxP sites [7,8] was then inserted between β-globin intron and polyA site of the expression vector. cDNA for murine Smad7 was derived from total RNA of FVB/NHSD mice by RT-PCR and inserted between 3'-loxP and 3'-polyA sites of the expression vector.

Screening-PCR for Smad7 single and Alb-Smad7 double transgenic mice

Screening-PCR for Smad7 transgenic mice was done on ear biopsies using REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich) using transgene-specific primer (S7-tg-for: ACAGCTCAATTGGGACAACA, S7-tg-rev: ATGCATGCCTGGAATCCC; product size 820 bp) detecting specifically Smad7-transgenic mice (S7-tg), whereas wildtype animals (WT) did not yield a PCR product. Screening-PCR for Alb-cre & Smad7 doubletransgenic mice was done using transgen-specific primer for Smad7 (S7-tg-for and S7-tg-rev) and cre (cre-for: AAGTTGAATAACCGGAAATGG, cre-rev: AGCTACACCAGAGACGGAAAT) to specifically identify Alb-cre and Smad7-transgenic mice, respectively. Interbreeding of Alb-cre transgenic with Smad7-transgenic mice resulted in an expected ratio of non-, single- and double-transgenic offsprings.

RT-PCR in reference to transgenic Smad7 expression

RNA from liver tissue was isolated and 1µg was used to perform reverse transcription. PCR was done using specific primers to detect transgene-specific Smad7 in Alb-Smad7 double-transgenic mice (Smad7-tg; RT-S7-tg-for: TCACCTTTCCTATCAACCCC, RT-S7-tg-rev: CGCTCCTTGAGTTTCTTGAG). As a control, β-actin was used (β-act-for: GTGGGCCGCCCTAGGCACCA, β-act-rev: TAGCCCTCGTAGATGGGCACA).

Recombination assessment of Alb-Smad7 double transgenic mice

Determination of recombination efficacy in Alb-Smad7 double-transgenic mice was done by lacZ-staining [9].

2. Hematological analysis, measurement of circulating iron and tissue non-heme iron levels

Through all the study we used female mice of 12 weeks of age. Mice were sacrificed by CO_2 inhalation. Heparin blood was collected to assess hematological parameters using Abc VetCcil instrument. Tissue non-heme iron content and iron deposition, revealed by Prussian blue staining on paraffin-embedded liver and spleen tissue sections, was done as previously described [10]

3. Immunohistochemistry

Paraffin-embedded liver specimens (of 4μm) were stained with Hematoxylin/Eosin, and with Smad7, phosphorylated Smad2, pico-Sirius red and α-SMA, as previously described [11] [12]

4. RNA isolation, Reverse-transcription and real-time PCR

Total RNA was isolated from the liver tissues using Trizol reagent (Invitrogen, USA) following manufacturer's instruction. RNA quality and quantity was controlled using the Nanodrop 2000 system (Thermo Scientific, USA). RevertAid H Minus (M-MulV) reverse transcriptase (Fermentas, USA), 5x RT reaction buffer, random primers (200ng/μl, Thermo Scientific, USA) and 10mM dNTPs were used to convert 1-2μg of RNA to cDNA following the manufacturer's instructions. Quantitative real time PCR was carried out in 10 μl of reaction volume using SYBR Green I Dye (Invitrogen, USA) on ABI ViiA-7 system (Applied Biosystems, USA). The mRNA abundance of the gene of interest was calculated relative to the expression of the reference gene *Gapdh* as previously described [13]. Primers used in the study are:

Primer Name	**Forward Sequence (5'-3')**	**Reverse Sequence (5'-3')**
Activin	GGGCCTTTTGATGCTGTCG	TGGCAGAATGGTCTCTTGCAG
Bmp6	GTGACACCGCCACAAC	TCGTAAGGGCTCTCTG
Col4a1	CTGGCGGTTCAGGTCCAAT	TTCCAGGCAATCCACGAGC
Gapdh	CCCATTCTCGGCCTTGACTGT	GTGGAGATTGTTGCCATCAACGA
Hamp	ATACCAATGCAGAAGAGAAGG	AACAGATACCACACTGGGAA
Id1	ACCCTGAACGGCGAGATCA	TCGTCGGCTGGAACACATG
Pai-1	TGCATCGCCTGCCATTG	CTTGAGATAGGACAGTGCTTTTTCC
Smad7	GCAGGCTGTCCAGATGCTGT	GATCCCCAGGCTCCAGAAGA
SnoN2	TGCGTCCCAGTCTAAAGAGG	GCACACAGCAGACTCAGATTTC
Tgfβ1	CCGCAACAACGCCATCTATG	CTCTGCACGGGACAGCAAT

5. Protein isolation and Western blot analysis

Protein extracts were prepared after homogenization of the livers in RIPA lysis buffer (50mM Tris-Cl pH 8.0 /150mM NaCl /1% NP-40 /0.5% DOC /0.1% SDS) complemented with the protease inhibitors (Complete Mini (25) ROC 11836153001, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (1mM Na3O4Va /25mM NaF /1mM PMSF, Sigma Aldrich, Germany) for 30 minutes on ice [13]. Protein concentration, SDS-polyacrylamide gel electrophoreses, and the immunoblotting with Smad1/5/8, Smad1 (all from Cell Signaling Technology, USA; 1:1000) and with anti-β-actin (Sigma Aldrich, USA; 1:10,000) was performed as described in [13]. The signals were quantified by scanning densitometry and computer-assisted image analysis (ImageJ; www://rsb.info.nih.gov/ij/).

6. Statistical analyses

Data were analyzed using GraphPad Prism software and results are shown as mean ± SEM (standard error of mean). Due to the small sample size, a non-parametric distribution was assumed. For the statistical analysis the Mann-Whitney-U test for pairwise comparisons was used. A probability value $p<0.05$ was considered statistically significant.

7. References to Supplemental Material and Methods

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