

Growth differentiation factor 11 attenuates liver fibrosis via expansion of liver

Supplementary figures, tables and methods

Supplementary figure legends

Supplementary Fig. 1. GDF11 is mainly expressed by hepatic stellate cells in the liver. (a) The western blot analysis of mouse aSMA in fibrotic livers. (b) The qPCR analysis of *Gdf8* in whole liver, purified hepatocytes, non-parenchymal liver cells (NPC) and embryonic (E13.5) liver. (c-d) qPCR analysis for human *GDF8* mRNA expression in patients with fibrosis and normal liver control. HEK-293T RNA was used as positive control for human *GDF8* expression. (e) The qPCR-based analysis and immunofluorescence staining in various purified liver cells from healthy mice such as hepatocytes (HC), hepatic stellate cells (HSC), Kupffer cells (KC) and liver sinusoidal endothelial cells (LSEC). (f) The qPCR analysis of *Gdf11* in various liver cells from healthy liver. (g) The qPCR analysis of mouse *Gdf11* in purified HSC from control and fibrotic mice. (h) The western blot analysis of aSMA and GDF11 in purified HSC from control and fibrotic mice. Data are mean \pm s.e.m.; two-tailed student's t-test (panel a, b and g) or two-sided Welch's t-test (panel c and d).

Supplementary Fig. 2. *In vivo* transduction of liver by AAV8 vectors.

Representative pictures showing the AAV.CMV.Cre targeting efficiency in mouse liver. Cre recombinase delivery in hepatocytes leads to expression of GFP due to excision of Tomato and a stop codon upstream of GFP in mTmG mouse model.

Supplementary Fig. 3. GDF11 does not induce fibrosis in other organs. (a,e)

Schematic of the experiments in CCl₄- (a-d) and DDC- (e-h) fibrosis mouse models. **(b,f)** Measuring the GDF11 content by ELISA in mouse serum. **(c,g)** Representative pictures showing the Sirius red staining on heart, lung, kidney, muscle, brain, intestine and spleen. Quantification of staining is shown adjacent to Sirius red staining images. Scale bars, 100 μ m. **(d,h)** qPCR analysis for *Acta2* expression in different organs. The experiments were repeated twice. Data are mean \pm s.e.m.; two-tailed student's t-test.

Supplementary Fig. 4. Recombinant GDF11 protects livers from fibrosis *in vivo*.

(a,f) Schematic of the experiments in CCl₄- (b-e) and DDC- (g-j) fibrosis mouse models (n=6 mice per group for both CCl₄ and DDC model). **(b,g)** Measuring the total collagen content by hydroxyproline assay in 1mg per kg body weight rGDF11 injected and control mice. **(c,h)** Representative pictures of HE, Sirius red and desmin stainings. Scale bars, 100 μ m. **(d,i)** Quantification of Sirius red and desmin stainings shown in panel c and h. For each mouse, 6 liver sections were stained in batches, pictures from 12 random fields per section were captured and quantified in a blinded manner using Image-J. **(e,j)** The qPCR-based analysis of *Acta2*, *p75NTR*, *Col1a1* and *Col2a1*. The experiments were repeated twice. Data are mean \pm s.e.m.; two-tailed student's t-test.

Supplementary Fig. 5. GDF11 promotes murine liver progenitor cell expansion

in ex vivo cultured organoids. (a) Schematic of the experimental design. The LGR5⁺ liver progenitor cells from mouse liver were sorted by FACS and seeded in 3D matrigel to generate organoids. **(b)** Serial images showing the outgrowth of a single LGR5⁺ liver progenitor cell. **(c)** Representative confocal images of a liver

progenitor cell organoid stained for KRT19, SOX9, HNF4A. Nuclei were counterstained with DAPI. Scale bars, 100 μ m. **(d)** Representative images of organoids treated with rGDF11 and control, and quantification of organoid numbers at day 4. For each well, all areas with organoids were imaged by serial pictures and quantified in a blinded manner (n=10 wells). **(e)** The qPCR analysis of *Lgr5*, *Prom1* and *Hnf4a* mRNA expression in GDF11-treated organoid. **(f)** The qPCR analysis of *Lgr5*, *Prom1* mRNA in organoids upon different concentrations of GDF11 treatment. **(g)** The qPCR analysis of *Gdf11* mRNA expression in sorted LGR5+ liver progenitor cells. Experiments were repeated twice for b-c, and three times for d-e. Data are mean \pm s.e.m.; two-tailed student's t-test.

Supplementary Fig. 6. Gene expression analyses of LGR5+ LPCs and engraftment analyses of transplanted LGR5+ LPCs. **(a)** The qPCR analysis of LGR5+ liver progenitor cells from AAV.control and AAV.GDF11 vector injected mice. **(b)** Schematic of the experimental design. The LGR5+ liver progenitor cells from mouse liver were sorted by FACS and transduced with lenti-CMV-GFP virus, followed by transplantation. **(c)** The engraftment of lenti-CMV-GFP transduced LGR5+ liver progenitor cells at day 7 after transplantation. Data are mean \pm s.e.m.; two-tailed student's t-test.

Supplementary Fig. 7. Characterization of human myofibroblasts *in vitro*. **(a)** Immunofluorescence staining of α SMA and Ki67 in human primary hepatic myofibroblasts. **(b,c)** The schematic and qPCR analysis of *ACTA2*, *DES* and *COL1A1* mRNA expression in myofibroblasts cultured in 2D or 3D Matrigel after 4 days.

Supplementary Fig. 8. Human LGR5+ cells suppress myofibroblasts activation *in vitro*. (a) Schematic of the experimental design. (b) Representative images of organoids co-cultured with myofibroblasts or treated with GDF11 neutralizing antibody at day 4. (c) The quantification of organoid number. For each well, all areas with organoids were imaged by serial pictures and quantified in a blinded manner (n=10 wells). (d) The qPCR analysis of *LGR5* and *PROM1* mRNA expression in organoids co-cultured with myofibroblasts or treated with GDF11 antibody at day 4. (e) Albumin ELISA performed with culture medium collected from human liver organoids that were either treated with GDF11 antibody or isotype. (f) The qPCR analyses of *ACTA2* and *COL1A1* mRNA expression in myofibroblasts co-cultured with LGR5+ cells. The LGR5+ cells from human liver organoids were sorted by FACS and co-cultured with myofibroblasts. (g) The LGR5+ cells from human liver organoids were sorted by FACS and treated with rGDF11 for 24 hours and subsequently co-cultured with myofibroblasts. The qPCR analysis of *ACTA2* and *COL1A1* mRNA expression in myofibroblasts co-cultured with GDF11 pre-treated LGR5+ cells after 24 hours. Data are mean \pm s.e.m.; two-tailed student's t-test.

Supplementary Fig. 9. GDF11 does not induce fibrosis in normal mice *in vivo*. (a) Schematic of the experiments. (b) The qPCR analysis for *Gdf11* in mice injected with AAV8-GDF11 or AAV.control (n=5 mice per group). (c) Representative pictures of HE staining and Sirius red stainings 98 days after AAV-GDF11 vector injection. Scale bars, 100 μ m. (d) The qPCR analysis for fibrogenic genes such as *Acta2*, *p75NTR*, *Col1a1* and *Col2a1*. (e) The FACS analysis for LGR5+ cells in non-injected and AAV.control and AAV.GDF11 vector injected BALB/c mice. The values shown are the mean of three independent experiments.

Supplementary Fig. 10. *In vitro* modulation of GDF11 in hepatic myofibroblasts.

(a,e) Immunofluorescence staining of GDF11 in murine and human myofibroblasts. (b,f) qPCR analyses showing expression of *Gdf11* in murine and human myofibroblasts either transfected with plasmid encoding for GDF11 or transfected with siRNA. (c,g) WST assay showing cell viability upon overexpression or inhibition of GDF11. (d,h) The qPCR analyses of *Acta2* and *Col1a1* mRNA expression upon overexpression or inhibition of GDF11. Data are mean \pm s.e.m.; two-tailed student's t-test.

Supplementary Fig. 11. The expression of LGR5 and GDF11 is upregulated in fibrotic livers.

(a,b) The qPCR analyses of human *LGR5* mRNA in patients with fibrosis (n=6) and controls (n=6) from Hannover Medical School, Germany (a) and patients with fibrosis (n=26) and controls (n=6) from HaiKou Hospital, China (b). (c,d) The qPCR analyses of human *GDF11* and *LGR5* mRNA in patients with different grades of fibrosis stage, F0 (n=6), F2 (n=7) and F4 (n=5) from HaiKou Hospital, China. Data are mean \pm s.e.m.; two-tailed student's t-test or two-sided Welch's t-test.

Supplementary Fig. 12. GDF11 promotes LGR5+ cells in NASH models.

(a) The qPCR analyses for human *GDF11* mRNA in NAFLD patients with fibrosis (n=6) and controls (n=6) from HaiKou Hospital, China. (b) The qPCR analyses for *Gdf11* in mice on HFD and subsequently injected with AAV8.GDF11 or AAV.control (n=6 mice per group). (c) Measuring the GDF11 content by ELISA in mouse serum. (d) Representative pictures of Sirius red staining in AAV.Control and AAV.GDF11 injected NASH mouse model. Scale bars, 100 μ m. (e) The FACS analysis for LGR5+ cells in AAV.control and AAV.GDF11 injected NASH mouse model. Data are mean \pm s.e.m.; two-tailed student's t-test (panel b and c) or two-sided Welch's t-test (panel a).

Supplementary table 1a: Human fibrotic liver samples obtained from Hannover Medical School, Germany

Patient number	Age	Nationality	Etiology
1	67	Germany	Alcoholic liver disease
2	70	Germany	Alcoholic liver disease
3	65	Germany	Alcoholic liver disease
4	31	Germany	Hepatoblastoma
5	37	Germany	HCC
6	51	Germany	PSC

Supplementary table 1b: Human fibrotic liver samples obtained from Haikou Hospital, China

Patient number	Age	Nationality	Etiology	Patient number	Age	Nationality	Etiology
1	31	China	HBV	14	43	China	NAFLD
2	33	China	HCV	15	45	China	HCV
3	33	China	HBV	16	46	China	HCV
4	35	China	HBV	17	46	China	HBV
5	35	China	HCV	18	46	China	HBV
6	36	China	HBV	19	49	China	HBV
7	37	China	NAFLD	20	52	China	HBV
8	37	China	HBV	21	53	China	NAFLD
9	39	China	NAFLD	22	53	China	NAFLD
10	39	China	HBV	23	55	China	HBV
11	39	China	HBV	24	57	China	HBV
12	40	China	HCV	25	57	China	HCV
13	40	China	NAFLD	26	59	China	HBV

Supplementary table 2. List of antibodies

Epitope	Company	Catalogue Number	Dilution	Application
LGR5	Abcam	ab75732	1/50	IF and Flow Cytometry
HNF4A	Santa Cruz	SC-6556	1/200	IF
Ki67	Thermo Fisher Scientific	RM-9106-S1	1/400	IF
BrdU	Abcam	ab6326	1/400	IF
PHH3	Cell Signaling	9701	1/300	IHC-P
CD133	eBioscience	11-1331-82(13A4)	1/50	IHC-P/IF
DESMIN	Thermo Fisher Scientific	RB-9140	1/200	IHC-P/IF
CD31	Abcam	ab56299	1/100	IHC-P/IF
F4/80	Abcam	ab6640	1/200	IHC-P/IF
SOX9	Millipore	ab5535	1/300	IF
KRT19	DSHB	Ab2133570	1/200	IF
MUP	Santa Cruz	Sc21856	1/200	IF
GDF11	R&D	MAB19581	1/500	Western and ELISA
Goat anti-rat conjugated with Alexa-594	Thermo Fisher Scientific	A-11007	1/400	IF
Goat anti-rabbit conjugated with Alexa-594	Thermo Fisher Scientific	R37117	1/400	IF and Flow Cytometry
Goat anti-Rat IgG conjugated with Alexa-647	Thermo Fisher Scientific	A-21247	1/400	IF
Goat anti-rabbit conjugated with Alexa-488	Thermo Fisher Scientific	A-11008	1/400	IF and Flow Cytometry
Goat anti-rat conjugated with Alexa-488	ThermoFischer Scientific	A-11006	1/400	IF

Supplementary table 3. List of primers

Official Gene Symbol	RefSeq Accession number	forward 5'-3'	reverse 5'-3'
Human			
GDF11	NM_005811.3	Hs00195156_m1 taqman probe (Thermo Fisher)	
GDF8	NM_005259.2	Hs00193363_m1 taqman probe (Thermo Fisher)	
ACTB	NM_001101.3	Hs99999903_m1 taqman probe (Thermo Fisher)	
HNF4a	NM_000457.4	Hs00604438_m1 taqman probe (Thermo Fisher)	
SOX9	NM_000346.3	Hs01001343_g1 taqman probe (Thermo Fisher)	
KRT19	NM_002276.4	Hs00761767_s1 taqman probe (Thermo Fisher)	
LGR5	NM_001277226.1	GACTTTAACTGGAGC ACAGA	AGCTTTATTAGGGAT GGCAA
ACTB	NM_001101.3	CTCTTCCAGCCTTCC TTCCT	AGCACTGTGTTGGCG TACAG
Mouse			
Gdf11	NM_010272.1	Mm01159973_m1 taqman probe (Thermo Fisher)	
Gdf8	NM_010834.2	Mm01254559_m1 taqman probe (Thermo Fisher)	
Gapdh	NM_001289726.1	Mm99999915_g1 taqman probe (Thermo Fisher)	
Lgr5	NM_010195.2	Mm01251798_g1 taqman probe (Thermo Fisher)	
Prom1	NM_001163577.1	Mm00477124_m1 taqman probe (Thermo Fisher)	
Hnf4a	NM_008261.2	Mm00433964_m1 taqman probe (Thermo Fisher)	
Gapdh	NM_001289726.1	TGCCCCCATGTTTGT GATG	TGTGGTCATGAGCCC TTCC
Sox9	NM_011448.4	GTGCAAGCTGGCAA GTTGA	TGCTCAGTTCACCGA TGTCC
Krt19	NM_001313963.1	CGGACCCTCCCGAG ATTACA	TGGAGTTGTCAATGG TGGCA
Col1a1	NM_007742.4	TAGGCCATTGTGTAT GCAGC	ACATGTTTCAGCTTTGT GGACC
Col2a1	NM_001113515.2	AGCAGGTCCTTGAA ACCTT	AAGGAGTTTCATCTG GCCCT
Acta2	NM_007392.3	GTTCAAGTGGTGCCTC TGTC	ACTGGGACGACATGG AAAAG
P75NTR	NM_033217.3	TACAGTAGCCTGCCC CTGAC	GTCTATATGCTCCGG CTGGT
Cd31	NM_001032378.2	TCCCACCGAAAGCA GTAAT	CCCACGGAGAAGTAC TCTGTCTATC
F4/80	NM_001355722.1	CTTTGGCTATGGGCT TCCAGTC	GCAAGGAGGACAGAG TTTATCGTG
Pck1	NM_011044.3	TGCCCCAGGCAGTG AGGAAGTT	GTCAGTGAGAGCCAG CCAACAGT
G6pc	NM_008061.4	CGACTCGCTATCTCC AAGTGA	CGACTCGCTATCTCC AAGTGA

Supplementary methods

GDF11 treatment

Recombinant GDF11 protein (rGDF11) was purchased from Peprotech (cat.:120-11) and reconstituted in water as 100 µg/ml stock solution. rGDF11 was added to medium freshly every second day for *in vitro* experiments. Murine or human organoids were seeded in equal numbers in matrigel, treated with rGDF11 every second day and quantified at day 4. For the co-culture of primary human myofibroblasts with human organoids, we collected and mixed human myofibroblasts with human organoids, then seeded them in Matrigel. To overexpress or knockdown GDF11 in murine or human myofibroblasts, AAV.GDF11 plasmid or siGDF11 (Qiagen) were transfected into cells using lipofectamine 3000 or Lipofectamine RNAiMAX reagent (ThermoFisher), respectively. For the *in vivo* experiments, rGDF11 (0.1mg/kg) was injected into mice via intraperitoneal injection. The AAV-GDF11 viral particles were injected into mice via the portal vein. We used a specific antibody against GDF11 to detect its protein levels (R&D systems, Catalog number: MAB19581).

Generation of AAV vectors

cDNAs with the mouse *Gdf11* and the human *GDF11* ORF were purchased from Biocat (Germany). We cloned the mouse *Gdf11* ORF downstream of a CMV promoter into the pAAV plasmid. The *Lgr5* promoter construct was purchased from Genecopoeia (MPRM41028-LvPM03). The diphtheria toxin A ORF (Addgene, cat: 58536) was cloned under the transcriptional control of *Lgr5* promoter in the AAV plasmid. AAV8 vector particles were produced in 293T cells. Briefly, we transfected 293T cells with pDP8.ape (Plasmid Factory, Germany) and pAAV-CMV-GDF11 by

calcium phosphate method, collected and purified virus after 72 hours. Similarly, AAV2 vectors were prepared to express diphtheria toxin A from the *Lgr5* promoter. Viral vector titers were determined by qPCR using primers targeting *Gdf11* or DTA. pAV-GDF11-shRNA was purchased from Vigene Biosciences (SH821896). AAV.NGF.GDF11 and AAV.NGF.shGDF11 vectors, which are designed to preferentially target hepatic stellate cells and myofibroblasts by binding to p75NTR receptor, were prepared to express GDF11 or shRNA from the CMV promoter. Viral vector titers were determined by qPCR using primers targeting CMV.

Mouse models of liver fibrosis and NASH

To establish CCl₄ induced liver fibrosis model, mice received intraperitoneal injections of 10% CCl₄ in olive oil (Sigma), twice per week for 8 weeks together with metamizol supplementation in drinking water for pain control. To induce cholestatic liver fibrosis, mice were fed on DDC diet for 8 weeks. To establish NASH model, the 8-12 weeks mice were fed either a Surwit high-fat diet (HFD) (E15771, Ssniff, Germany) with 60 kcal% from fat plus 0.5% (w/w) extra cholesterol for 14 weeks.

Tissues for organoid culture

Four following lines of human liver organoids were derived from distant non-tumor normal tissues.

1 (Labeled as H1): Liver segment resection due to HCC. The patient had cirrhosis due to ethanol consumption). The tumor distant normal liver tissue was used for organoid culture.

2 (Labeled as H3): Surgery for liver adenomatosis. The healthy normal liver tissue was used for organoid culture.

3 (Labeled as H4): Surgery for Klatskin tumor (central cholangiocarcinoma), The distal normal liver tissue was used for organoid culture.

4 (Labeled as H9): Surgery for central liver hemangioma. The healthy distal tissue was used for organoid culture.