Supplemental Methods

Antibodies and reagents

A rabbit polyclonal anti-α_9 antibody was used for immunohistochemistry and immunoblotting. Other antibodies used were $\alpha_9\beta_1$ (for adhesion blockade; clone Y9A2, Abcam), α_5 (for immunoblotting, Millipore; for adhesion blockade and immunostaining, clone HMα5-1, Santa Cruz), α_v (for immunoblotting, clone 21/CD51, BD Bioscience; for adhesion blockade, clone H9.2B8, Santa Cruz), peptidylprolyl isomerase B (cyclophilin B, Thermo Scientific), αSMA (clone 1A4, Sigma), vinculin (Sigma), desmin (clone DE-U-10, Sigma), elastin (Accurate Chemical), total FN (for Western immunoblotting, clone 10/Fibronectin, BD Bioscience; for immunostaining and adhesion blockade, clone C-20, Santa Cruz), and EIIIA (for adhesion blockade, Millipore; for adhesion blockade and immunostaining, clone IST9, Santa Cruz).

Integrin knockdown

shRNAs targeting rat integrin subunits α_9, α_v, and α_5 were cloned into adenoviral vectors using the BLOCK-iT U6 RNAi Entry Vector Kit and BLOCK-iT Adenoviral RNAi Expression System (Invitrogen). Virus was made in 293A packaging cells, amplified, and titered. An MOI of 25, which demonstrated no cell toxicity, was used for all experiments. shRNA sequences are listed in Supplemental Table 1.

Transwell chemotaxis assays

Hepatic stellate cells were differentiated in culture and then plated in 0.5% serum containing media in the upper chamber of 8.0 μm pore FluroBlok transwell inserts (BD Bioscience) coated with 0.02 mg/ml pFN or cFN on both sides. Cells were incubated at 37°C for 19 h, then stained with 2 μg/ml calcein AM (Invitrogen, Molecular Probes) for 1 h. Fluorescence intensity from the bottom side of the insert was measured using a microplate reader (BioTek Synergy 2) and corrected for background fluorescence. Results were normalized to fluorescence measured on an uncoated insert. For some experiments hepatic stellate cells were also visualized on an inverted microscope (model DM IRB; Leica Microsystems) with images taken using a CoolSNAP camera and analyzed using iVision software. There were 3-5 replicates per condition per experiment.

Time-lapse microscopy

Images were taken at 10-minute intervals for 6 hours on a Nikon TE300 inverted microscope (Nikon Instruments, Inc.) in a custom incubation chamber. Video files were assembled and analyzed with Image-Pro Plus software (Media Cybernetics, Inc.). For quantification of area, perimeter, and circularity, cells were traced in every 4^{th} frame (at 40 minute intervals), and these parameters were measured using NIH ImageJ.

Western blotting

Stellate cells were removed from hydrogels with trypsin and lysed in total lysis buffer (0.15 M NaCl, 0.5 M EDTA pH 8.0, 1% Triton-X 100, 10 mM Tris HCl pH 7.4) supplemented with Complete protease inhibitor (Roche), 0.1% SDS, 100 nM PMSF, and 0.5% deoxycholic acid. Peptidylprolyl isomerase B (1:10,000, Thermo Scientific) was used as a loading control. IRDye infrared fluorophore conjugated secondary antibodies were purchased from Li-Cor. Membranes were visualized using the Odyssey Infrared Imaging System (Li-Cor). Antibodies used were against α_9 (1:10,000), α_5 (1:1,000), α_v (1:500), and αSMA (1:10,000).

qRT-PCR

Cells were homogenized on QiaShredder columns (Qiagen), and total RNA was purified using the RNeasy kit (Qiagen). Pieces of liver were homogenized in Trizol reagent (Invitrogen) using the Bullet Blender (Next Advance). Total RNA was quantified by spectrometry, and the same amount of RNA per sample was used in each experiment. Super Scriptase III (Invitrogen) was used for reverse transcription. qRT-PCR with Fast SYBR green (Applied Biosystems) was performed on an ABI StepOne Plus Real-Time PCR System (Applied Biosystems). Results were quantified using the relative standard curve method. Expression was normalized to control transcripts, as stated in each figure legend. 3 technical replicates were used per sample. Primer sequences are listed in Supplemental Table 2.

Confocal microscopy

Stellate cells cultured on 12 kPa hydrogels were fixed in 4% paraformaldehyde, permeabilized with 0.1% TritonX-100, and stained with primary antibody overnight at 4°C. Primary antibodies used were αSMA (1:1,000) and vinculin (1:500). Secondary antibodies were Cy2 donkey anti mouse IgG2a and Cy5 donkey anti mouse IgG1 (Jackson Immunolabs). Images of stained stellate cells were obtained using a Zeiss LSM-510 Meta confocal microscope and processed with LSM Image Browser.

TGFβ1 treatment

Portal fibroblasts were cultured in the presence of 100 pM TGFβ1 (R&D Systems) or 100 nM of TβR1 inhibitor (Calbiochem) or vehicle control.

PDGF-BB treatment

Hepatic stellate cells were cultured for 24 hours in the presence of 100 ng/ml PDGF-BB (R&D Systems) or 10 μM PDGFRβ inhibitor, AG1295 (Calbiochem), or vehicle control.

Serum alanine amino transferase (ALT) measurement

50-250 μl whole blood was collected from mice at the time of euthanization by cardiac puncture. Blood was centrifuged to separate serum from erythrocytes. Serum ALT activity (U/L) was measured on a BioTek Synergy 2 microplate reader using an NADPH-based

absorbance assay, according to the manufacturer's instructions (Stanbio Laboratory). A normal serum ALT activity is < 38 U/L.

Sirius red staining

Formalin-fixed paraffin-embedded liver sections were incubated for 1 hour at room temperature in 0.1% Direct Red 80 (Sigma) in picric acid, then washed and dehydrated.

Scoring of fibrosis

Degree of fibrosis was scored by a blinded observer using slides stained with sirius red. We used a scoring system of 0-4, in which 0 represents no fibrosis, 1 represents mild fibrosis (some portal or sinusoidal fibrotic expansion), 2 represents moderate fibrosis (much portal or sinusoidal fibrotic expansion but few bridges or septa), 3 represents severe fibrosis (numerous bridges or septa), and 4 represents cirrhosis.

Hydroxyproline measurement

Liver pieces (50-200 mg) were homogenized in 6 N HCl and incubated at 110° C for 24-36 hours. The resulting slurry was incubated in chloramine T solution (1.45% chloramine T, 10% n-propanol, and 0.5 M sodium acetate, pH 6.0) for 20 minutes at room temperature. Ehrlich's solution (1M p-dimethylaminobenzaldehyde, 70% n-propanol, and 30% perchloric acid) was then added, and the mixture was incubated for 15 minutes at 65° C. Absorbance at 555 nm

was measured on a BioTek Synergy 2 microplate reader, and hydroxyproline content in samples was quantified by comparing to absorbance values for a standard curve composed of serial dilutions of trans-hydroxy-proline. Results are expressed as total μg hydroxyproline per liver.

Immunofluorescence on frozen tissue

Liver pieces were flash frozen in O.C.T. (Sakura) in liquid nitrogen then sectioned. Slides were warmed to room temperature, then blocked with StartingBlock Blocking Buffer (Thermo Scientific). Primary anti-desmin antibody (diluted 1:100 in PBS supplemented with 0.1% BSA and 0.2% Triton X-100) was applied overnight at 4° C. Cy3-conjugated secondary antibody (Jackson Immunolabs) was diluted 1:200 and applied for 30 minutes at 37° C. Slides were mounted with Fluorescent Mounting Media (KPL) and visualized on a Nikon E600 microscope. Images were taken with a CCD camera and IP Lab software.

Elastin staining

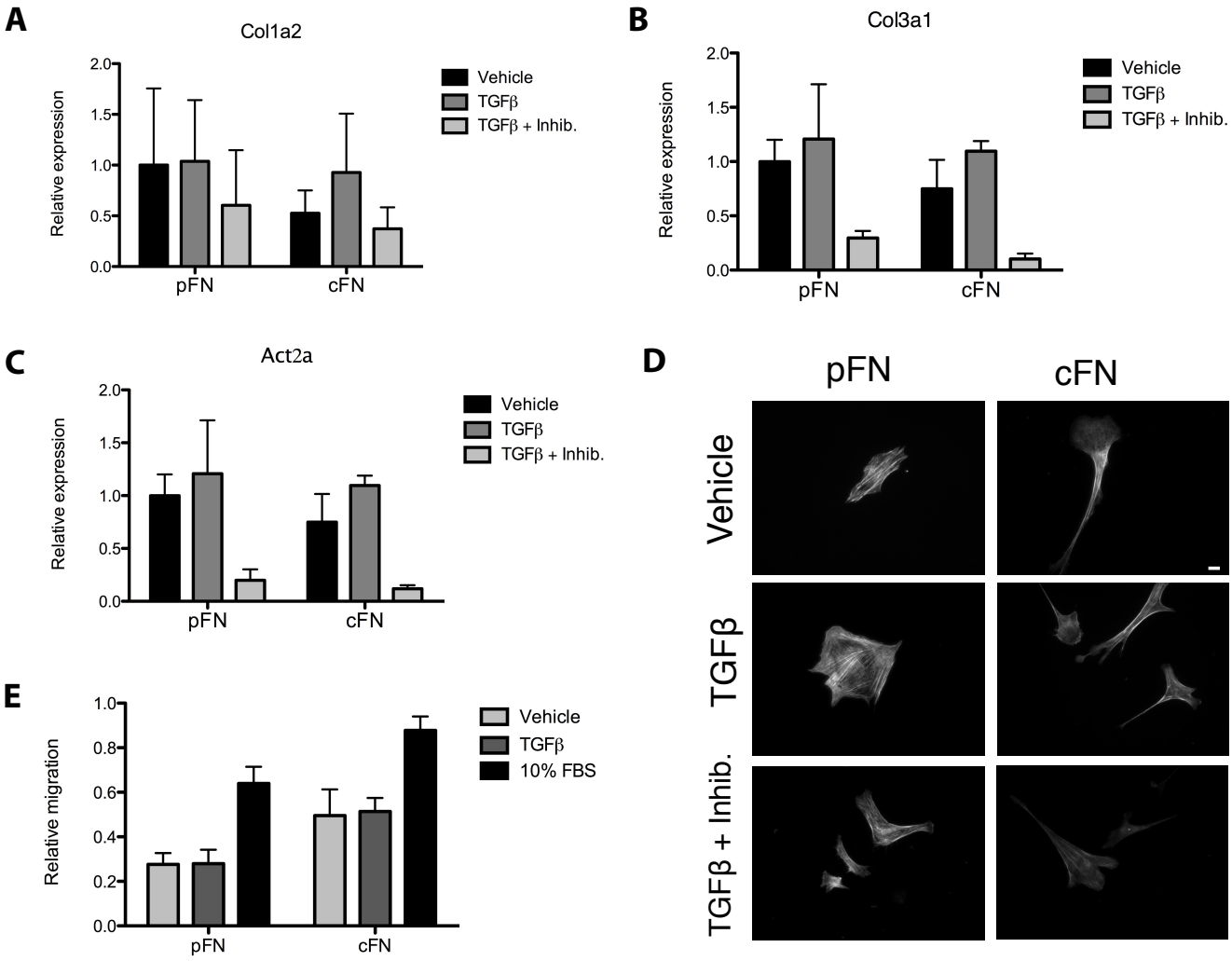
Formalin-fixed paraffin-embedded liver sections were dewaxed, then incubated in 0.5% hyaluronidase in TBS for 60 minutes at room temperature. Primary anti-elastin antibody was diluted 1:100 in TBS with 3% BSA and 1% NGS and applied overnight at 4° C. Slides were blocked in TBS supplemented with 3% BSA and 0.9% NaCl for 20 minutes at room temperature. Endogenous peroxidase was quenched, and biotinylated secondary anti-rabbit antibody diluted 1:200 in TBS was applied for 30 minutes at 37° C.

Integrin	Sequence 5' → 3'
Itga5	GCAGATCTCGGAGTCCTATTACGAATAATAGGACTCCGAGATCTGC
Itgav	GGCTTGAATTCGGTGCCATCTCGAAAGATGGCACCGAATTCAAGCC
Itga9	GGACGCATCCAACTTCATTCACGAATGAATGAAGTTGGATGCGTCC
Scramble	GCATATTCACGAGTATGTGCCCGAAGGCACATACTCGTGAATATGC

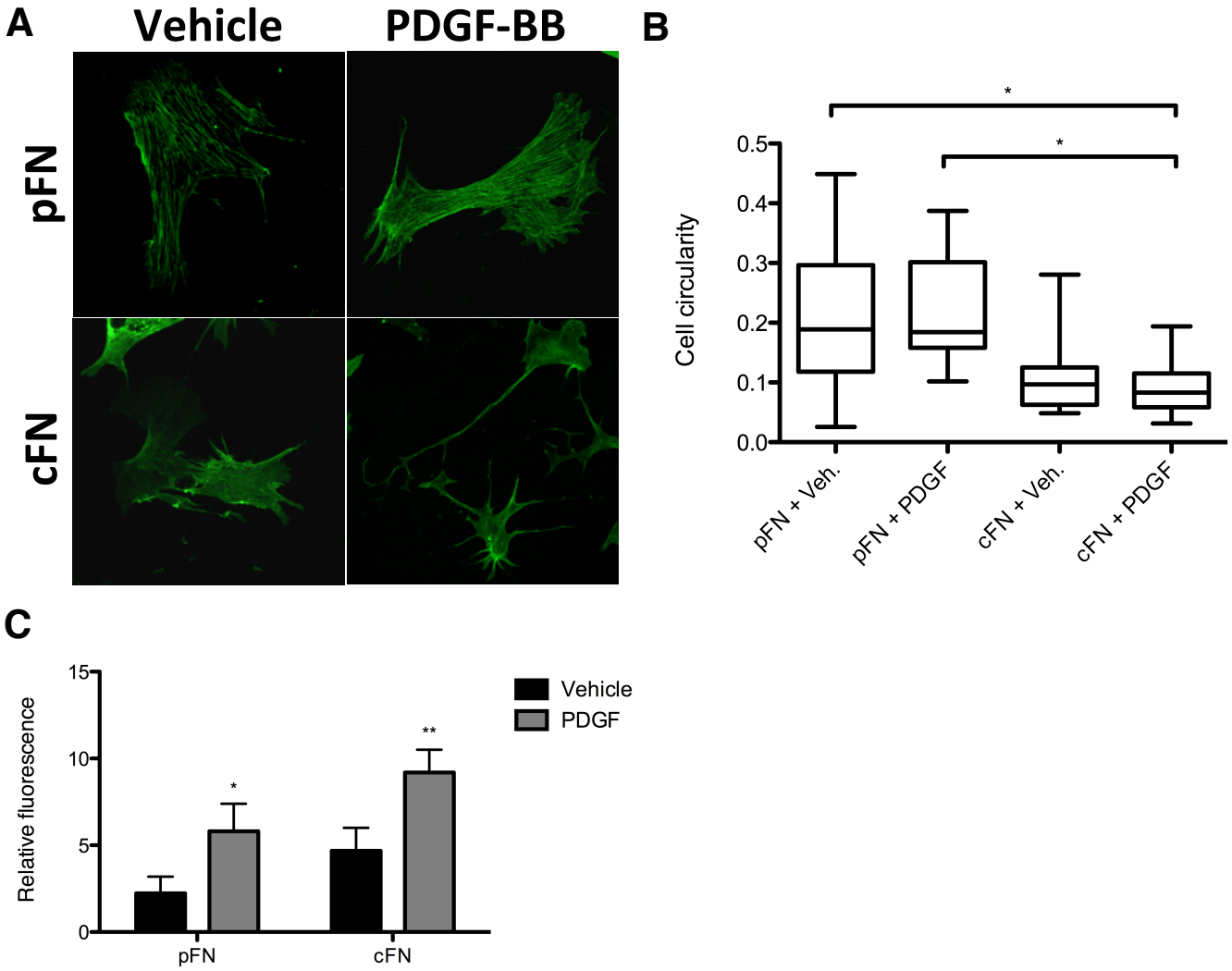
Supplemental Table 1: Integrin-targeting shRNA sequences. Three shRNA sequences were screened per integrin and the most effective one used. Sequences were designed to target mouse and rat, but not human, integrin subunits.

Protein name	Gene name	Species	Forward primer 5' → 3'	Reverse primer 5' → 3'
α_4 integrin	Itga4	Rat	CCCAGGCTACATCGTTTTGT	ATGGGAGTGAGGATGTCTCG
α_5 integrin	Itga5	Rat	GCAGCTTCCCCAAAAGAAAC	TGTAGAGGACGTAGATGAGCAG
α_9 integrin	Itga9	Rat	CACTGGTGCATGAAGTGGAC	TGTTGTAGACCTGGAGAGTG
αSMA	Acta2	Mouse	ATTGTGCTGGACTCTGGAGATGGT	TGATGTCACGGACAATCTCACGCT
αSMA	Acta2	Rat	TGTGCTGGACTCTGGAGATG	GAAGGAATAGCCACGCTCAG
α_v integrin	Itgav	Rat	GCTGCCGTTGAGATAAGAGG	TGCCTTGCTGAATGAACTTG
β2 microglobulin	B2m	Mouse	TGGTCTTTCTGGTGCTTGTC	GGGTGGAACTGTGTTACGTAG
Collagen I	Col1a2	Mouse	TTCTCCTGGCAAAGACGGACTCAA	AGGAAGCTGAAGTCATAACCGCCA
Collagen I	Col1a2	Rat	TTCTCTACTGGTGAAACCTGC	ACCCCTTCTGCGTTGTATTC
Collagen III	Col3a1	Mouse	CCCCCAGGACCTACTGGCCC	GACCACGCCCACCGGGAAAG
Collagen III	Col3a1	Rat	GAAGTCTCTGAAGCTGATGGG	GGCCTTGCGTGTTTGATATTC
Collagen IV	Col4a1	Rat	AGTTCCCCGCCGTCTCTGTT	ACAATCACCCTTCGCAGCGG
EIIIA+ cFN	n/a (splice variant of Fn1)	Mouse	AGTCAGTGTGGTTGCCTTG	CTGAACACTGGGTGCTATCC
EIIIB+ cFN	n/a (splice variant of Fn1)	Rat	ATACCGTCATCCCAGAGGTG	TCATAGTCAATGCCGGGTTC
EIIIB+ cFN	n/a (splice variant of Fn1)	Mouse	AGATGACAAGGAAAGTGCCC	GGTGGAAGAGTTTAGCGGG
Elastin	Eln	Mouse	AGTTCCTGGTGTTGGTCTTC	CCTTGGCTTTGACTCCTGTG
Fibronectin (total)	Fn1	Mouse	CTTTGTGGTCTCATGGGTCTC	AGCAGGTCAGGAATGTTCAC
Fibronectin (total)	Fn1	Rat	TCCATTCCACCTTATAACACCG	AACGATGCTTCCTGAGTCTG
Glucuronidase β	Gusb	Mouse	CACCCCTACCACTTACATCG	ACTTTGCCACCCTCATCC
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Mouse	CTTTGTCAAGCTCATTTCCTGG	TCTTGCTCAGTGTCCTTGC
n/a (rRNA)	Rn18s	Mouse and rat	CCCAGTAAGTGCGGGTCATAA	GATCCGAGGGCCTCACTAAAC
Tenascin C	Tnc	Rat	TGACACCACCTCCTACAGCCTG	TCCAAACCCAGTGGCATAGGCCT
TGF-β1	Tgfb1	Mouse	CCTGAGTGGCTGTCTTTTGA	CGTGGAGTTTGTTATCTTTGCTG
TIMP-1	Timp1	Mouse	GGACCTGGTCATAAGGGCTA	TTTTAGCATCTTAGTCATCTTGATCTT

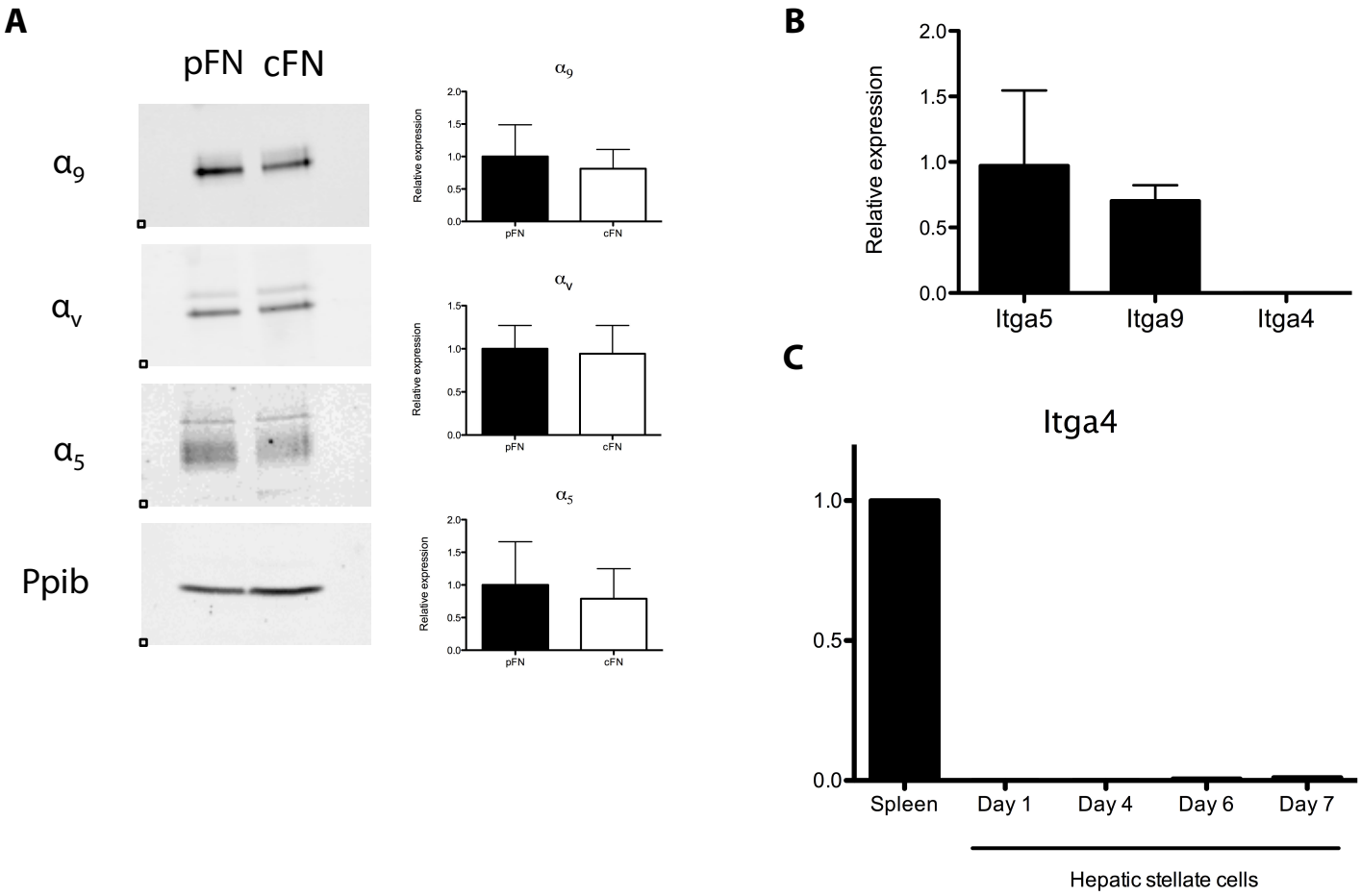
Supplemental Table 2: qRT-PCR primer sequences. Primer pairs were designed in different exons using Integrated DNA Technologies SciTools RealTimePCR program.



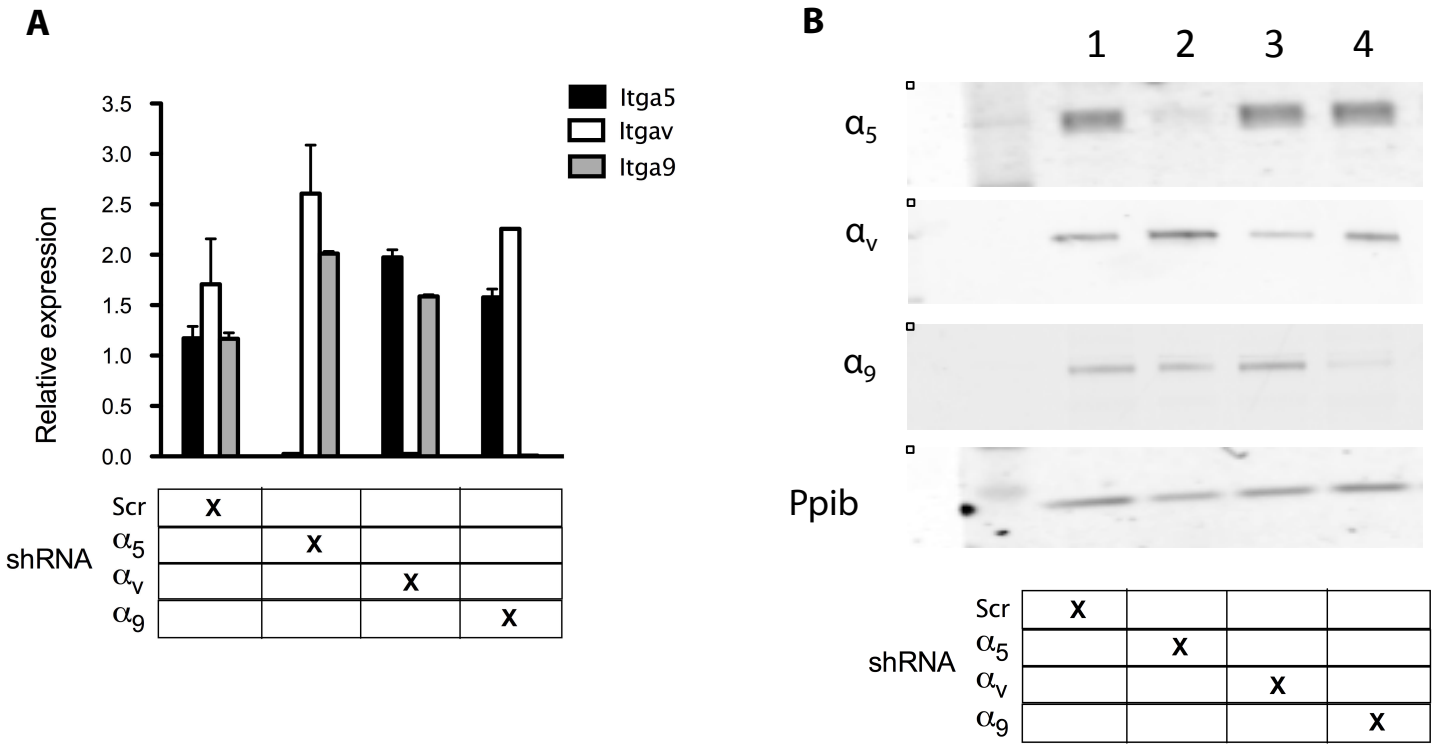
Supplemental Figure 1. Effects of EIIIA+ cFN are independent of TGFβ. A-D. Hepatic stellate cells were cultured on 12 kPa pFN-coated or cFN-coated polyacrylamide gels. Beginning 24 hours after plating cells were treated with vehicle control, 100 pM TGFβ, or 100 pM TGFβ and 100 nM TβR1 kinase inhibitor for the duration of the experiment. **A-C.** qRT-PCR was performed on day 7. Col1a2 = collagen I, Col3a1 = collagen III, Act2a = αSMA. Results are averaged from 3 independent experiments. **D**. On day 7 cells were stained for αSMA. Scale bar = 50 μm. **E.** Stellate cells were cultured on tissue culture plastic for 6 days, starved overnight in media with 0.5% serum, then plated on pFN-coated or cFN-coated transwell inserts and induced to migrate toward 100 pM TGFβ or 10% serum.



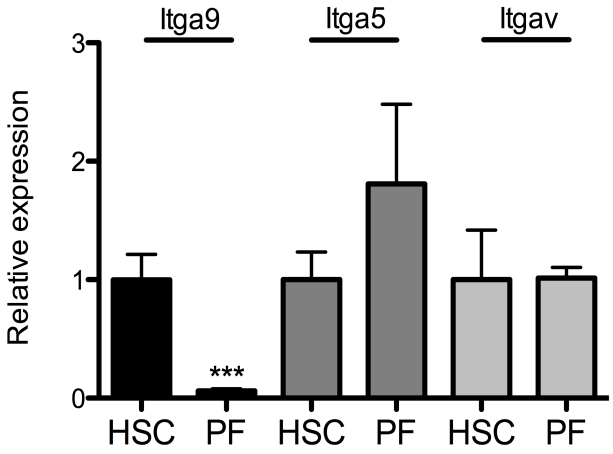
Supplemental Figure 2. PDGF-BB promotes chemotaxis on both pFN and cFN. A. Hepatic stellate cells were cultured on 12 kPa pFN-coated or cFN-coated polyacrylamide gels. After 6 days in culture regular growth media was replaced with media containing only 0.5% FBS. The next day cells were treated with 100 ng/ml PDGF or vehicle control, for 24 hours, then fixed and stained for αSMA. **B.** Cell circularity was calculated using NIH Image J. Results are averaged from 2 independent experiments. * = $p < 0.05$. **C**. Stellate cells were cultured on plastic for 6 days, starved overnight, then plated on pFN-coated or cFN-coated transwell inserts and induced to migrate toward 100 ng/ml PDGF. Results are averaged from 5 independent experiments. * or ** = significant over vehicle control.



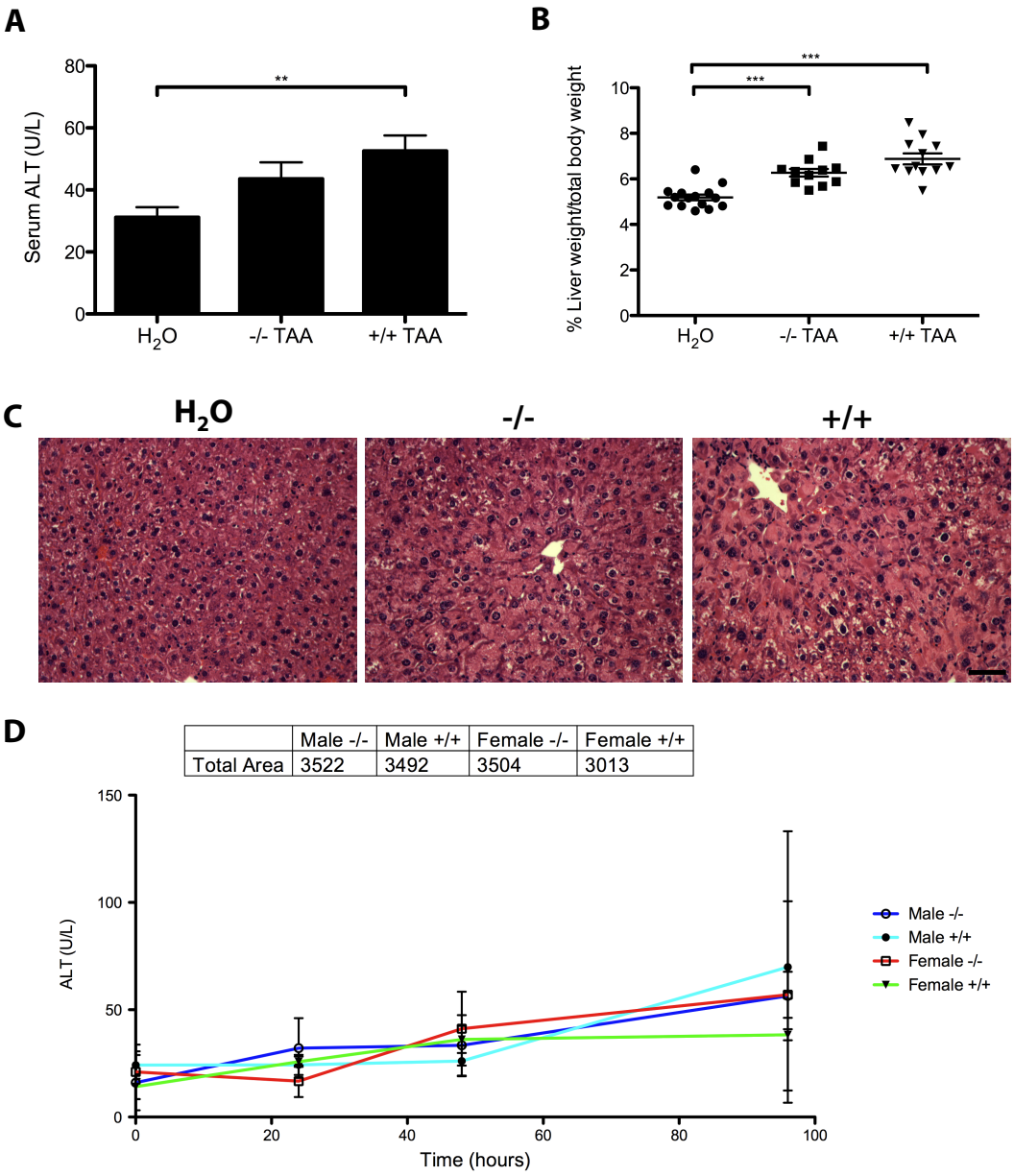
Supplemental Figure 3. Integrin expression in hepatic stellate cells. A. Cells were cultured for 7 days on pFN-coated or cFN-coated gels, and expression of α_5, α_9, and α_v was detected by immunoblotting (left) and quantified by measuring fluorescence intensity of bands normalized to peptidylprolyl isomerase B (Ppib) loading control (4 independent experiments; right). **B.** qRT-PCR on day 7 hepatic stellate cells. Cells express α_5, α_9, and α_v (not shown), but not α_4. **C.** To confirm that pre-myofibroblastic hepatic stellate cells also lack α_4, qRT-PCR for α_4 was performed on hepatic stellate cells at day 1, 4, 6 or 7 after isolation. Whole spleen lysate served as a positive control. Results are normalized to expression of 18s.



Supplemental Figure 4. Integrin knockdown is effective. Four days after isolation, hepatic stellate cells were transduced with 25 MOI of adenovirus expressing shRNA targeting one of three integrin subunits or with a scrambled control. 72 hours post-transduction, integrin expression levels were measured by qRT-PCR **(A)** or immunoblotting **(B).** Error bars in **(A)** represent standard deviation from technical replicates, and results shown are representative of 4 independent experiments.

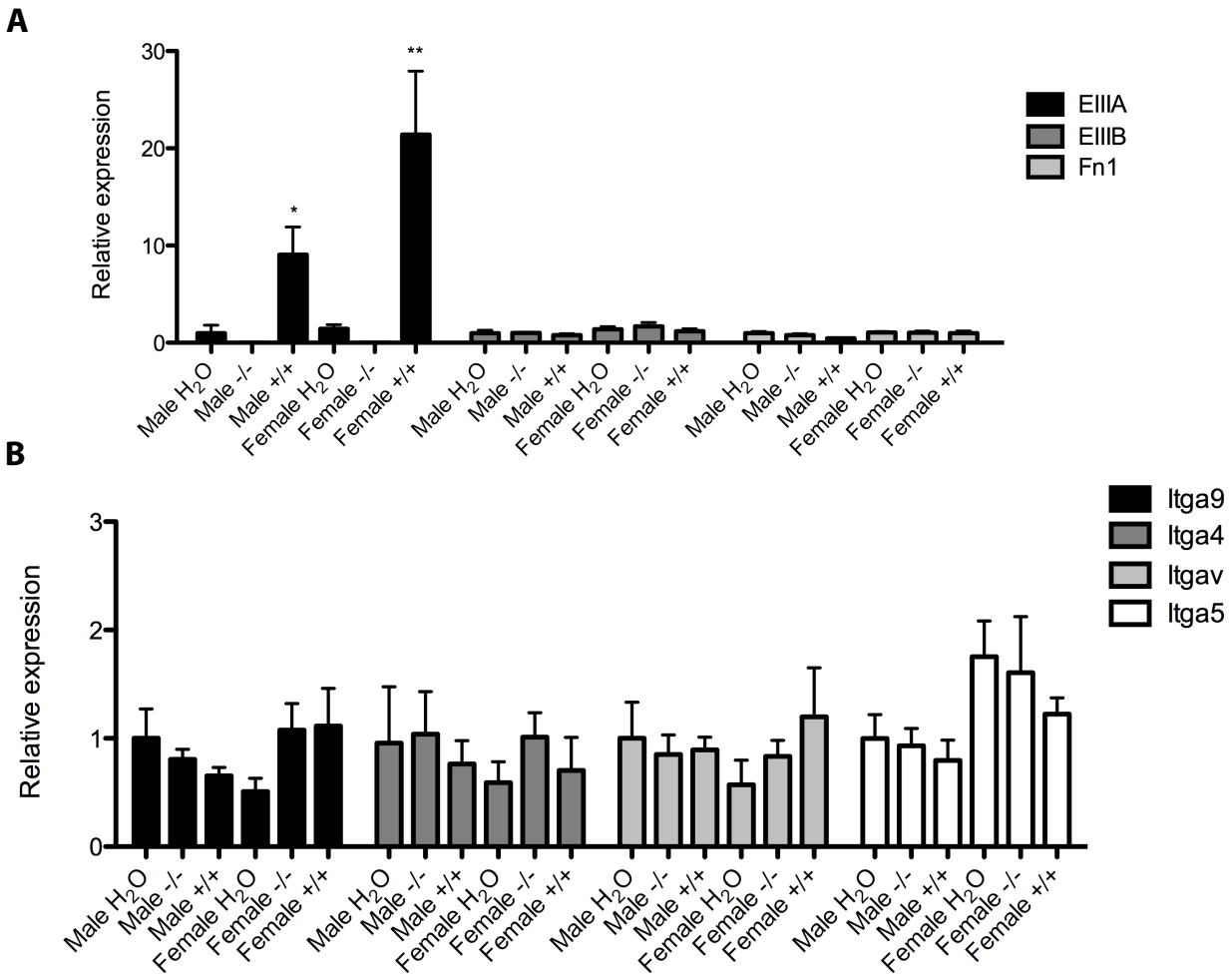


Supplemental Figure 5. Portal fibroblasts express little α_9. Integrin expression was measured by qRT-PCR in hepatic stellate cells (HSC) or portal fibroblasts (PF) after 7 or 10 days of culture, respectively. Itga9 = α_9, Itga5 = α_5, and Itgav = α_v. Results are normalized to expression of 18s and are averaged from 3 independent experiments. p-values were calculated by Student's t-test. *** = $p \leq 0.005$.

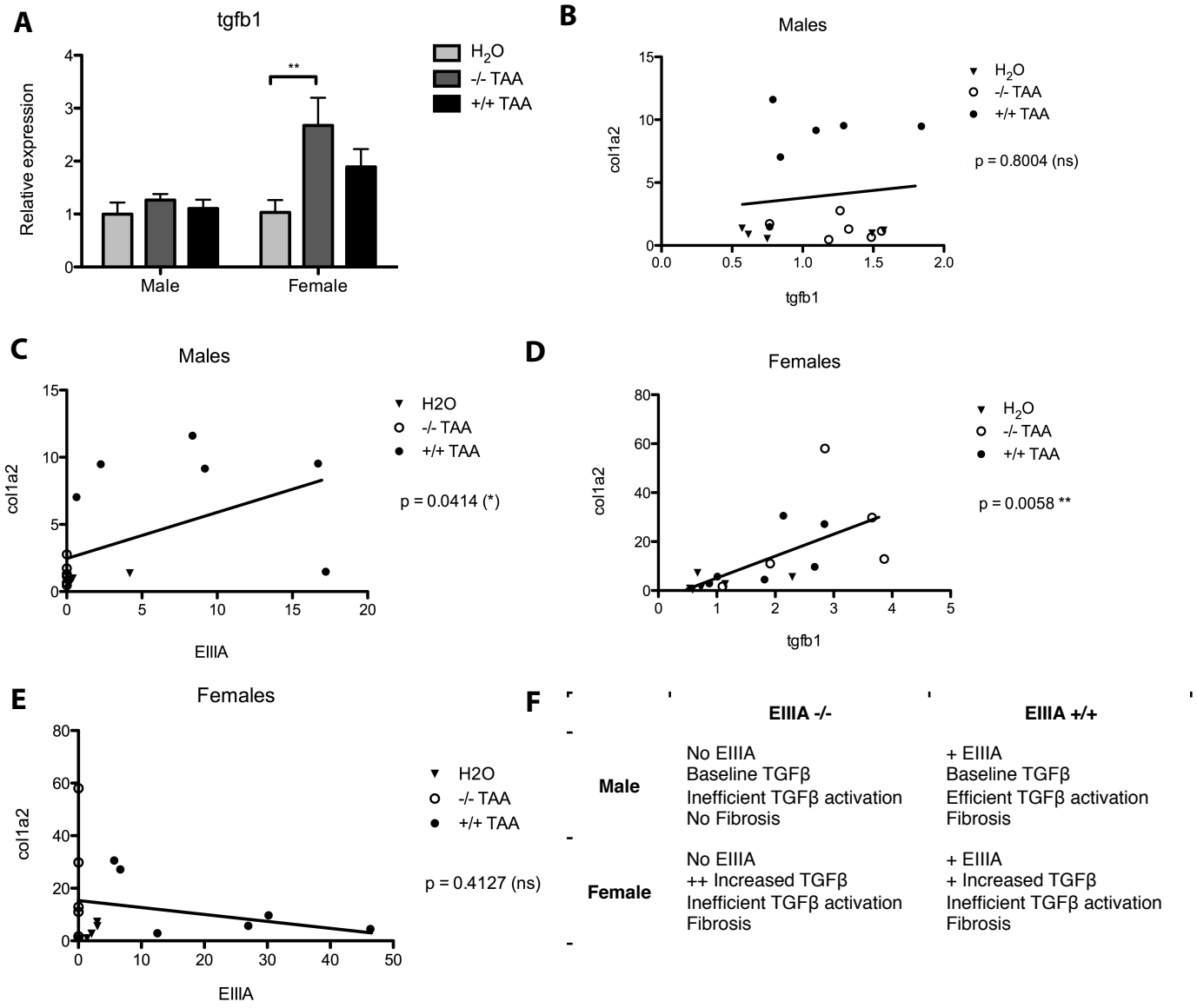


	Male -/-	Male +/+	Female -/-	Female +/+
Total Area	3522	3492	3504	3013

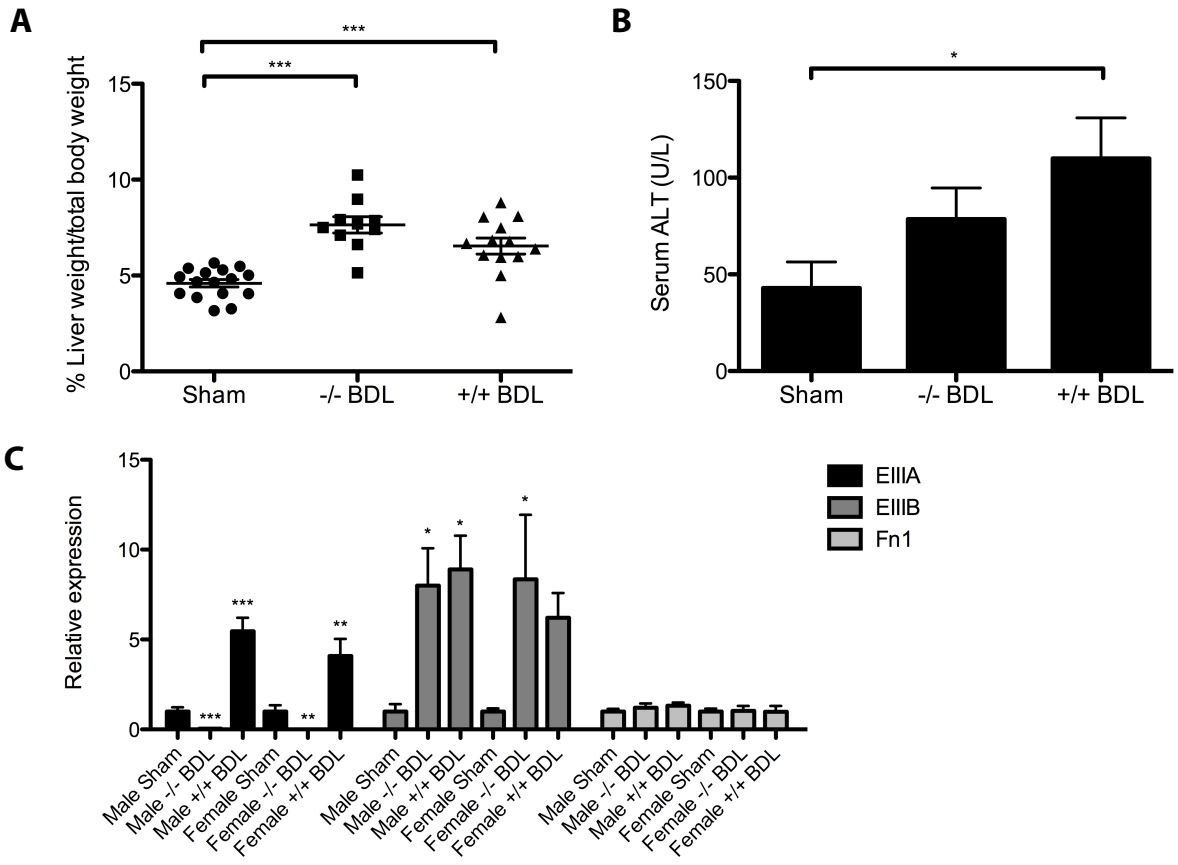
Supplemental Figure 6. EIIIA$^{-/-}$ and wild type mice develop similar extent of liver injury after 12 weeks of thioacetamide (TAA). Mice were administered 200 mg/L TAA in their drinking water for 12 weeks. n = 13 H_2O treated control mice of mixed genotype with respect to EIIIA, 11 EIIIA$^{-/-}$ mice, and 12 wild type mice. **A.** Serum ALT activity (U/L) was measured. **B.** Liver weight as a percentage of total body weight was calculated. Statistical significance was performed by one way ANOVA with Bonferroni's post test. * = $p \leq 0.05$. ** = $p \leq 0.01$, *** = $p \leq 0.005$. **C.** Liver sections were stained with hematoxylin and eosin (H&E) following 12 weeks of TAA. Representative sections are shown. Scale bar = 50 µm. **D.** Serum ALT activity (U/L) was measured at 0, 24, 48, or 96 h after initial TAA administration. Area under the curve is shown in the table over the graph.



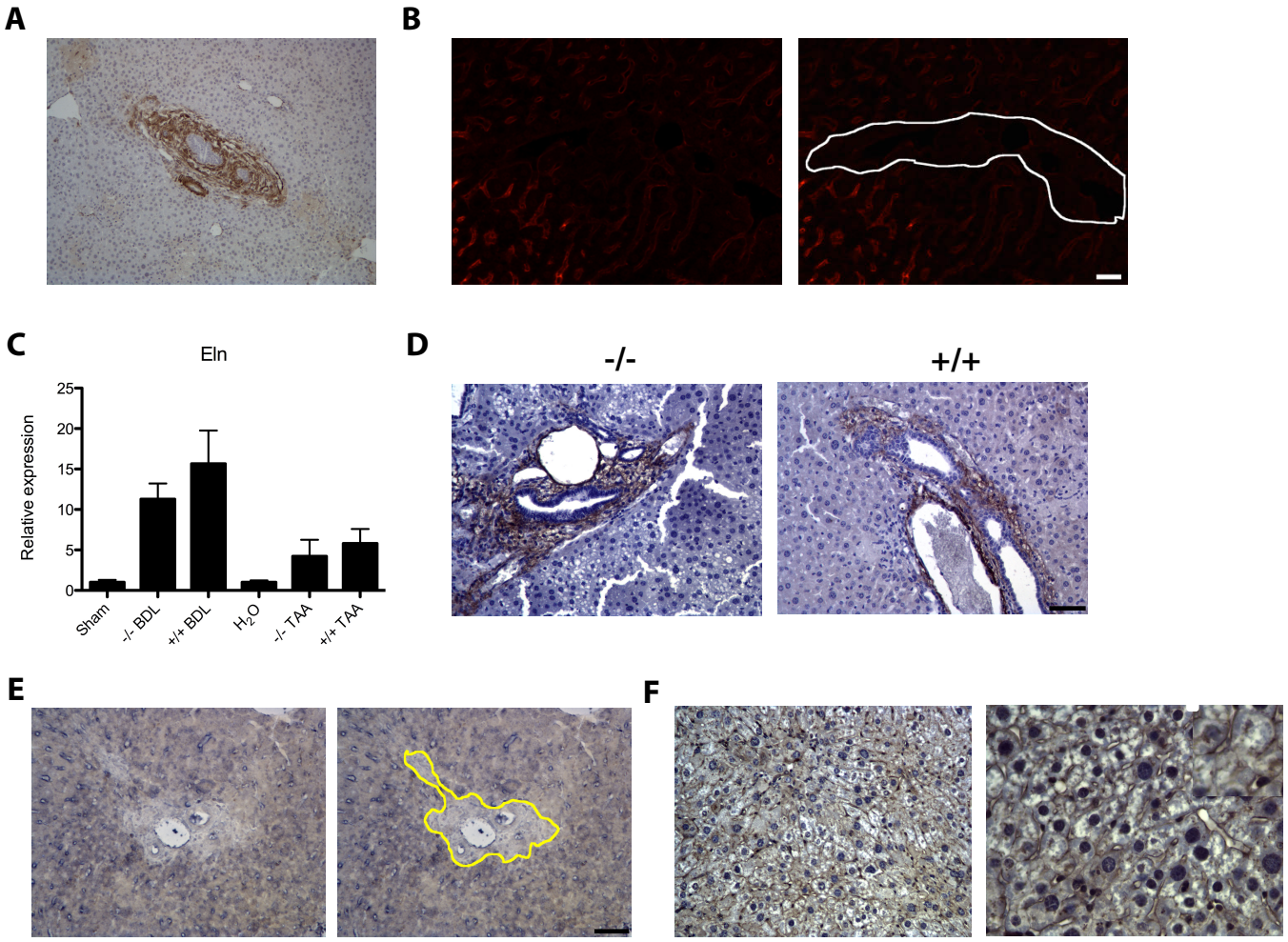
Supplemental Figure 7. EIIIA$^{-/-}$ and wild type mice have similar fibronectin and integrin expression. Mice were administered 200 mg/L TAA in their drinking water for 12 weeks. n = 5 male H_2O controls, 6 male EIIIA$^{-/-}$ mice, 6 male wild type mice, 6 female H_2O controls, 5 female EIIIA$^{-/-}$ mice, and 6 female wild type mice. **A.** The EIIIA and EIIIB fibronectin splice variant and total fibronectin (Fn1) were measured by qRT-PCR. Symbols indicate that EIIIA was statistically significant over sex-matched H_2O control. The difference in EIIIA expression between males and females is not statistically significant. **B.** α_9 (Itga9), α_4 (Itga4), α_v (Itgav), and α_5 (Itga5) were measured by qRT-PCR. Statistical significance was performed by one way ANOVA with Bonferroni's post test. * = $p \leq 0.05$. ** = $p \leq 0.01$.



New Supplemental Figure 8. Sex specific relationship between EIIIA and TGFβ. Mice were administered 200 mg/L TAA in their drinking water for 12 weeks. n = 5 male H_2O controls, 6 male EIIIA$^{-/-}$ mice, 6 male wild type mice, 7 female H_2O controls, 5 female EIIIA$^{-/-}$ mice, and 6 female wild type mice. **A.** qRT-PCR was performed for TGFβ-1 (tgfb1). **B-E.** Pearson correlation was calculated for expression of TGFβ (tgfb1) and Collagen 1 (Col1a2) (B-C) or for expression of EIIIA and Collagen I (Col1a2) (D-E). **F.** Table summarizing phenotypes and offering a potential model. In males, degree of fibrosis correlates with EIIIA expression, whereas in females degree of fibrosis correlates with TGFβ expression.



Supplemental Figure 9. Injury in EIIIA$^{-/-}$ mice and wild type mice after bile duct ligation. Mice underwent bile duct ligation and were euthanized 14 days post-operation. Depending on the experiment, n = between 6-16 sham operated control animals (10 male, 6 female) of mixed genotype, 12 EIIIA$^{-/-}$ mice (7 male, 5 female), and 13 wild type mice (7 male, 6 female). **A.** Percent liver weight to total body weight. **B.** Serum ALT activity (U/L). **C.** EIIIA, EIIIB, and total fibronectin (Fn1) were measured by qRT-PCR. *, **, and *** = statistically significant relative to sham control.



Supplemental Figure 10. αSMA-expressing cells in the portal scar are portal fibroblasts. A. αSMA staining (brown) from wild type mice 6 days post bile duct ligation. **B.** Desmin staining in livers of wild type mice 6 days post bile duct ligation. Portal area is outlined in white and image is pseudocolored red (right panel). Scale bar = 50 μm. **C**. Elastin (Eln) expression was measured by qRT-PCR in wild type or EIIIA$^{-/-}$ mice 2 weeks after bile duct ligation (BDL) or after 16 weeks of thioacetamide (TAA) treatment. **D.** Liver sections from day 6 post bile duct ligation were stained for elastin (brown) and with hematoxylin (blue). Scale bar = 50 μm. **E.** α_9 staining was performed on liver sections from wild type mice that underwent bile duct ligation**.** α_9 staining (blue) exhibits a sinuosoidal pattern and is excluded from the portal area (outlined by yellow line in right panel) with the exception of biliary epithelial cells. Scale bar = 50 μm. **F.** α_9 staining was performed on liver sections from wild type mice that received thioacetamide for 12 weeks. α_9 staining (brown) exhibits a sinuosoidal pattern. Sections are counterstained In hematoxylin (blue). Note that colors in (**E**) and (**F**) are opposite. Left panel of (**F**) is 20x magnification. Right panel is 40x magnification, and right panel inset is a further enlarged portion of the right panel.