Supporting Online Material for

Regulation of Hepatic Stellate Cell Differentiation by the Neurotrophin Receptor p75^{NTR}

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Materials and Methods

Animals

Plg-/- mice and *p75^{NTR}-/-* mice were obtained from The Jackson Laboratory. *Plg-/-* mice spontaneously develop liver disease characterized by fibrin deposition, hepatocyte necrosis, and HSC activation (*S1, S2*) . *Plg-/-* mice and *p75^{NTR}-/-* mice were bred to obtain a double heterozygous first generation (F_1). Double heterozygous F_1 mice were then bred to obtain *plg-/- p75^{NTR}-/-* mice. In experiments, *plg+/+ p75^{NTR}+/+*, *plg-/- p75^{NTR}+/+*, and *plg+/+ p75^{NTR}-/-* littermates were used as controls. Mice were fed standard chow and had access to food and water *ad libitum*. All animal procedures were performed under the guidelines set by the University of California San Diego Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Liver imaging and histology

Whole liver images were obtained by a Zeiss Stemi 2000-C stereoscope using an AxioCam HRc camera and AxioVision software (Carl Zeiss, Inc.). Liver cryosections (10 μm) were used for Hematoxylin staining and immunostaining.

For immunochemical detection of p75^{NTR}, liver cryosections were fixed in ice cold 4% paraformaldehyde (PFA) for 30 minutes, then rinsed with PBS three times. Peroxidase activity was quenched by incubation with 1.8% hydrogen peroxide in PBS for 10 minutes at room temperature. After rinsing three times with PBS, sections were blocked with antibody diluent (3% BSA/0.1% Triton X-100/PBS) + 3% goat serum for 30 minutes at room temperature. Slides were then rinsed again three times with PBS and incubated with primary antibody (rabbit anti-p75^{NTR}, 1:500, gift of M.V. Chao, New York University) in antibody diluent overnight at 4°C. Slides were washed 3 times for 5 minutes each in PBS, incubated with secondary antibody (biotin-conjugated goat anti-rabbit IgG, 1:300, Vector Laboratories) in antibody diluent for 30 minutes at room temperature, then washed with PBS 3 times for 5 minutes each. Bound antibody was visualized by using the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories) according to manufacturer's instructions with 3-amino-9-ethylcarbazole (AEC) (Sigma) as a chromogen.

For immunochemical detection of fibrin or desmin, liver cryosections were fixed in ice cold methanol for 7 minutes, then rinsed with PBS two times. For desmin immunostaining, the tissue was permeabilized with 0.1% Triton X-100/PBS for 1 hour at room temperature. After rinsing two times with PBS, sections were blocked with antibody diluent + 3% serum (horse for fibrin; goat for desmin) for 30 minutes at room temperature. Slides were incubated with primary antibody [sheep anti-fibrin(ogen), 1:100, US Biological; rabbit anti-desmin, 1:50, Santa Cruz Biotechnology] overnight at 4°C. For double confocal immunofluorescence rabbit anti-desmin (1:50) and goat anti-p75^{NTR} (1:100, Santa Cruz) were used. Slides were washed 3 times for 5 minutes each in PBS, incubated with secondary antibody [Cy3-conjugated donkey anti-sheep IgG; FITC-conjugated goat anti-rabbit IgG; Cy3-conjugated donkey anti-goat IgG; all 1:100, Jackson ImmunoResearch) in antibody diluent for 30 minutes at room temperature, washed again with PBS 3 times for 5 minutes each, and finally mounted with SlowFade Gold Antifade Reagent (Invitrogen) + DAPI (1 μg/mL). Images were obtained using an Axioplan 2 microscope (Zeiss) and AxioCam HRc camera.

To examine liver cell proliferation in vivo, mice were injected intraperitoneally (i.p.) with 100 mg/kg 5-bromo-2'-deoxyuridine (BrdU; Calbiochem) daily for 3 days and sacrificed on the fourth day. Cryosections were stained using the BrdU Immunohistochemistry System (Calbiochem) according to manufacturer's protocol.

Detection of fibrin in liver tissue

Fibrin was isolated from liver tissue and detected by western blot as described in Weiler-Guettler *et al.* (*S3*), with modifications. Fifty mg of snap-frozen liver tissue was homogenized in extraction buffer [10 mM sodium phosphate buffer, pH 7.5, with 0.1 M ε–aminocaproic acid (Sigma), 5 mM ethylene diamine tetraacetic acid (EDTA; Fisher), protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem; 1:100), and 10 U heparin/mL. After agitation at 4°C for 14 hours, particulate material (including fibrin) was collected by centrifugation at 10,000x*g* for 10 minutes, resuspended in extraction buffer without protease inhibitors, centrifuged again, and the final pellet was resuspended in 3 M urea. After agitation at 37°C for 2 hours, the samples were vortexed, then centrifuged at 14,000x*g* for 15 minutes. The sediment was dissolved in 2X reducing SDS sample buffer (120 mM Tris, pH 6.8, 20% glycerol, 4% SDS, and 200 mM DTT), subjected to SDS-PAGE (8% gel) and transferred to PVDF membrane (Immobilon-P, Millipore Corp.) by electroblotting. Fibrin β chains (~54 kDa) were detected with mouse anti-human fibrin antibody (mAb NYB T2G1, Accurate Chemical & Scientific Corp.; 1:500), followed by a peroxidase-labeled anti-mouse IgG and chemiluminescence system (ECL, Amersham Biosciences).

RNA extraction and real-time reverse transcription (RT) polymerase chain reaction (PCR)

RNA was isolated from liver tissue and cultured cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was reverse transcribed to cDNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems) according to manufacturer's instructions using random hexamer primers. Real-time PCR analysis was performed using the Opticon DNA Engine 2 (MJ Research) and the Quantitect SYBR Green PCR kit (Qiagen) using 1.5 μL of cDNA template in a 25 μL reaction. PCR efficiencies of the primers were calculated by serial dilution of template and no significant differences in efficiency were found between the target genes and the housekeeping genes. Results were analyzed with the Opticon 2 Software using the comparative C_T method as described (*S4*). Data were expressed as $2^{-\Delta\Delta C}{}_T$ for the experimental gene of interest normalized against the housekeeping gene and presented as fold change *vs.* the relevant control. The following primers were used:

αSMA (*S5*):	Fwd 5' AAC GCC TTC CGC TGC CC 3'
	Rev 5' CGA TGC CCG CTG ACT CC 3'
col1α1 (*S6*):	Fwd 5' CCT GCC TGC TTC GTG TAA ACT 3'
	Rev 5' TTG GGT TGT TCG TCT GTT TCC 3'
TGFβ-1 (*S7*):	Fwd 5' CCG CAA CAA CGC AAT CTA TG 3'
	Rev 5' GCC CTG TAT TCC GTC TCC TT 3'
GAPDH:	Fwd 5' CAA GGC CGA GAA TGG GAA G 3'
	Rev 5' GGC CTC ACC CCA TTT GAT GT 3'
HPRT (*S8*):	Fwd 5' GTT AAG CAG TAC AGC CCC AAA 3'
	Rev 5' AGG GCA TAT CCA ACA ACA AAC TT 3'

HSC isolation

Primary hepatic stellate cells were isolated as described in Schnabl *et al.* (*S9*) with modifications. Briefly, mice (2-4 months age) were anesthetized with 0.7 mL 2.5% Avertin injected i.p. and livers were perfused *in situ* through the inferior vena cava with warm perfusion solution [50 mL Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} (HBSS; Invitrogen)], followed by pronase solution [1 mg pronase (Calbiochem) per gram body weight in 40 mL DMEM/F12 medium (Invitrogen) + 1% penicillin/streptomycin (Invitrogen) + 0.25 μg/mL Amphotericin B (Invitrogen)], and finally collagenase solution [5 mg collagenase type IV (Sigma) per mouse in 50 mL DMEM/F12 + 1% penicillin/streptomycin + 0.25 μg/mL Amphotericin B]. After perfusions, the liver was removed and washed in warm HBSS. The livers of 3-4 mice were pooled, minced with scalpels, triturated with a 10 mL syringe, mixed with warm DMEM (Invitrogen) +1% penicillin/streptomycin + 0.25 μg/mL Amphotericin B to 35 mL, and shaken at 37°C for 10 minutes. The homogenate was then filtered through 2 layers of sterile gauze. Cold HBSS was added to 50 mL, centrifuged at 50x*g* at 4°C for 2 minutes, and the supernatant was collected. The pellet was washed 2 more times and supernatants collected. The supernatants were pooled and centrifuged at 500x*g* at 4°C for 7 minutes. The pellets were resuspended in cold HBSS, pooled, and mixed with 40% iodixanol (OptiPrep; Greiner Bio-One) in HBSS to achieve 17.5% iodixanol concentration. Five mL of 11.5% iodixanol in HBSS was layered on top of the cell solution, followed by 2 mL of HBSS. The gradient was centrifuged at 1400x*g* for 20°C for 17 minutes, and the HSCs were collected from between the HBSS layer and 11.5% iodixanol layer. HSCs were washed 3 times in 10 mL cold HBSS with centrifugation at 500x*g* at 4°C for 5 minutes. HSCs were resuspended in HSC medium [DMEM + 10% fetal bovine serum (FBS; Invitrogen) + 1% penicillin/streptomycin + 0.25 μg/mL Amphotericin B] and plated on 10 cm non-tissue culture-treated plates.

αSMA immunocytochemistry

HSC activation was assessed by immunochemical detection of αSMA, a cytoskeletal stress fiber that in the liver is only present in myofibroblasts, as described in Swaney *et al.* (*S10*), with modifications. HSCs (day 14-21, passage 2-4) were seeded on poly-D-lysine-coated (Sigma; 50 μg/mL) 8 well chamber slides (Nunc), 15,000 cells/well, and incubated for 2-3 days. Cells were washed with PBS, fixed with 4% PFA, permeabilized with 0.3% Triton X-100/PBS for 10 minutes, then blocked with 5% BSA/0.1% Triton X-100/PBS for 10 minutes. Slides were incubated with mouse anti-αSMA antibody (Sigma; 1:1000 in PBS) for 1 hour at 37°C, washed with 0.1% Triton X-100/PBS, and blocked as described above. FITC-conjugated anti-mouse IgG (Vector Laboratories; 1:200 in PBS) was added for 1 hour at 37°C, the slide was washed with PBS. For quantitation of percentage of activated cells, the total number of cells (DAPI+ nuclei) and αSMA+ cells were counted, with the % activated cells equal to the αSMA+ cells/total number of cells. For quantitation of activated cell size, the area of αSMA+ cells was calculated using AxioVision software (Carl Zeiss, Inc.).

Immunoblots

Wild-type and $p75^{NTR}$-/- HSCs (day 8, passage 2) were lysed with cell lysis buffer supplemented with protease inhibitor cocktail and the lysates were cleared by centrifuging at 13,000x*g* for 5 minutes. Protein concentration of cleared lysates was

determined by the Bio-Rad protein assay. Samples were prepared in 1X reducing SDS sample buffer (1 μg for αSMA; 20 μg for collagen I), then separated by SDS-PAGE electrophoresis (8% gel) and transferred to PVDF membrane by electroblotting. After blocking in 5% nonfat milk in TBS-T (25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM KCl, 1% Tween-20) for 1 hour at room temperature, membranes were incubated overnight with primary antibody diluted in 5% BSA in TBS-T (mouse anti-αSMA, 1:1000, Sigma; rabbit anti-collagen I, 1:1000, Rockland Immunochemicals; mouse anti-β tubulin, 1:1000, Sigma). Blots were washed 3 times for 5 minutes each with TBS-T, incubated with peroxidase-labeled secondary antibodies diluted in 5% nonfat milk in TBS-T for 1 hour at room temperature (goat anti-mouse IgG, 1:10,000, Santa Cruz Biotechnology; goat anti-rabbit IgG, 1:5000, Cell Signaling Technology), washed again, followed by detection with chemiluminescence (ECL, Amersham Biosciences).

Adenovirus-mediated gene expression in HSCs

Freshly isolated wild-type and *p75^{NTR}*-/- HSCs were seeded onto poly-D-lysine-coated 8 well chamber slides, 200,000 cells/well. The following day (day 1), the medium was changed to fresh HSC medium. On day 2, the cells were washed once with serum-free DMEM and then treated with adenovirus in DMEM + 2% FBS as follows: Ad p75^{NTR} FL (gift of P. Barker), containing the full length rat p75^{NTR} gene, 200 MOI (*S11*); Ad p75^{NTR} ICD (gift of P. Barker), containing the intracellular domain of the rat p75^{NTR} gene, 10 MOI (*S11*); Ad Rho (gift of J. Heller Brown), containing constitutively active RhoA (L63Rho), 10 MOI (*S12*); or Ad control (empty vector), 10 or 200 MOI. After 16 hours with adenovirus, the medium was changed to fresh DMEM + 10% FBS, and the medium was replaced every other day thereafter. On day 7, cells were stained for αSMA as described.

Lentivirus-mediated RNA interference in HSCs

To knockdown *p75^{NTR}* expression in wild-type HSCs, short hairpin RNA (shRNA) against mouse *p75^{NTR}* was expressed using a lentiviral vector system (BLOCK-iT™ Lentiviral RNAi Expression System; Invitrogen). Briefly, short hairpin DNA oligos (sequence depicted in fig. S4A) were synthesized based on a previously published small interfering RNA sequence against mouse *p75^{NTR}* (*S13*) and cloned into the pENTR/U6 Entry Construct vector. An LR recombination reaction was performed to insert the U6 promoter/mouse *p75^{NTR}* shRNA oligo/Pol III terminus cassette from the pENTR/U6 Entry Construct vector into the pLenti6/BLOCK-iT™-DEST vector. The vector generated, pLenti6/BLOCK-iT™ mouse *p75^{NTR}* shRNA vector, was cotransfected with ViraPower™ virus packaging mix using Lipofectamine™ 2000 (Invitrogen) into human embryonic kidney 293FT cells to produce virus. Supernatants containing lentivirus were harvested three days post-transfection and centrifuged at 3000 rpm for 5 minutes to pellet debris. Subsequently, 4 mL of supernatant was centrifuged at 150,000x*g* for 1.5 hours at 4°C to pellet virus, and the lentivirus pellet was resuspended in 150 μL of PBS. Freshly isolated wt HSCs were either seeded onto poly-D-lysine-coated 8 well chamber slides, 200,000 cells/well. The following day (day 1), the medium was changed to fresh HSC medium. On day 2, the cells were treated with lentivirus (20 μL in PBS) in DMEM + 10% FBS. After 24 hours with lentivirus, the medium was changed to fresh DMEM + 10% FBS, and the medium was replaced every other day thereafter. αSMA immunostaining was performed on day 10 as described.

Assessing the role of Trk and neurotrophins on HSC differentiation
Wild-type HSCs were isolated and seeded on 8 well chamber slides (100,000 cells/well) as described above. Media was changed and treatments were added every other day starting on day 1. Cells were treated with DMSO (Fisher), K252a, an inhibitor of Trk receptors (10 nM; Calbiochem), goat IgG (2 μg/mL; Jackson ImmunoResearch), goat anti-NGF, an NGF neutralizing antibody (2 μg/mL; Sigma), human IgG Fc fragment (1 or 20 μg/mL; Jackson ImmunoResearch), Fc-$p75^{NTR}$, a pan-neurotrophin scavenger (20 μg/mL; Alexis Biochemicals), or Fc-TrkB, a BDNF scavenger (1 μg/mL; R&D Systems). On day 7, cells were stained for αSMA and percentage of activated cells was quantitated as described.

Assessing HSC apoptosis in vitro
Wild-type and *$p75^{NTR}$*-/- HSCs (day 9-21, passage 2-4) were seeded in 96 well plates, 10,000 cells/well, and incubated overnight. The following day, the media was replaced with fresh HSC media with or without NGF (100 ng/mL; PeproTech). After a 24 hour incubation, apoptosis was assessed using the Cell Death Detection $ELISA^{PLUS}$ kit (Roche) according to manufacturer's instructions.

Assessing liver cell apoptosis in vivo
Liver cryosections were permeabilized with 0.1% Triton X-100 in TBS-T for 20 minutes at room temperature, then washed 3 times for 5 minutes each with TBS-T. Sections were incubated with CaspACE FITC-VAD-FMK In Situ Marker, a fluorescently-labeled pan-caspase inhibitor that irreversibly binds to activated caspases *in situ* (20 μM, diluted in TBS-T; Promega), for 1 hour at room temperature, and finally washed 5 times for 5 minutes each with TBS-T.

Phospho-cofilin immunocytochemistry
Rho activity in HSCs was assessed by immunochemical detection of phosphorylated cofilin, a downstream target in the Rho activation pathway. Cells were washed once with TBS, then fixed with cold methanol for 10 minutes at -20°C. Cells were washed 3 times for 5 minutes each with TBS, then incubated with freshly prepared 0.1% sodium borohydride in TBS for 5 minutes at room temperature. Cells were washed again 3 times for 5 minutes each with TBS, then blocking solution (10% goat serum, 1% BSA in PBS) was added for 1 hour at room temperature. Cells were washed once for 5 minutes with TBS, then incubated with rabbit anti-phospho-cofilin diluted in 1% BSA in TBS (1:50; Cell Signaling Technology) overnight at 4°C. After washing 3 times for 5 minutes each with TBS, cells were incubated with Cy3-conjugated donkey anti-rabbit IgG diluted in 1% BSA in TBS (1:200; Jackson ImmunoResearch) for 30 minutes at room temperature, and finally washed 3 times for 5 minutes each with TBS.

TAT-Pep5 treatment
Freshly isolated wild-type HSCs were seeded onto 8 well chamber slides ($1.5 – 2.5 \times 10^5$ cells/well) and treated every day with TAT-Pep5 (1 μM) (*S14*) or vehicle (DMSO) for 7 days. HSC activation was examined by αSMA staining and Rho activation was examined by phospho-cofilin staining as described.

HGF ELISA

The amount of HGF in liver samples was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Institute of Immunology, Tokyo, Japan) according to the manufacturer's instructions and as described in Yamada *et al.* (*S15*). Briefly, 0.2-0.6 g of frozen liver tissue was homogenized on ice in 4 volumes of extraction buffer (20 mM Tris buffer, pH 7.5, 2 M NaCl, 0.1% Tween-80, 1 mM EDTA, and 1 mM PMSF) and then centrifuged at 19,000x*g* for 30 minutes at 4°C. The supernatants were collected, diluted 1:4 with sample diluent (extraction buffer without NaCl) and passed over a HiTrap Heparin column (Amersham Biosciences) to bind HGF. The HGF-enriched fractions were eluted with extraction buffer, and 50 µL was applied to the HGF ELISA.

Hepatocyte isolation, co-culture with HSCs, and hepatocyte proliferation assay

For co-culture experiments, wild-type or $p75^{NTR}$-/- HSCs (day 7-21, passage 2-4) were seeded onto cell culture inserts (24-well format, PET membrane with 0.4µm pore size; BD Biosciences), 10,000 cells/insert, one day prior to hepatocyte isolation (*S16*). Primary hepatocytes were isolated from 2-4 month old wild-type C57BL/6 mice (Harlan Sprague Dawley) as described in Galijatovic *et al.* (*S17*), with modifications. Briefly, mice were anesthetized with 2.5% Avertin injected i.p. The inferior vena cava was canulated and the portal vein cut. The liver was perfused *in situ* with HBSS (Ca^{2+} and Mg^{2+}-free) containing 0.5 mM EGTA and 10 mM Hepes at pH 7.4, followed by perfusion with collagenase solution [1 mg/mouse Liberase Blendzyme 3 (Roche) in HBSS, with Ca^{2+} and Mg^{2+}]. The liver was removed and washed with cold HSC medium. Hepatocytes were isolated by mechanical dissociation with forceps, filtered through a sterile 70 µm filter, and washed twice by centrifugation at 50x*g* for 2 minutes. Hepatocytes were cultured in 24-well plates coated with collagen type I from calf skin (Sigma), ~30,000 cells/well, in 0.5 mL of HSC medium. Four hours after plating, the hepatocytes were washed twice with co-culture medium (DMEM + 0.2% FBS + 1% penicillin/streptomycin + 0.25 ug/mL Amphotericin B), and then incubated in 0.7 mL co-culture medium with or without HGF (R&D Systems; 50 ng/mL). Concurrently, HSCs in inserts were washed twice with co-culture medium, incubated in 0.2 mL co-culture medium, and transferred to the hepatocyte-containing wells. After 24 hours, [*methyl*-^{3}H]Thymidine (Amersham Biosciences) was added to the hepatocyte medium (1 µCi/mL). Co-cultures were incubated for 48 hours total and hepatocyte proliferation was assessed by [*methyl*-^{3}H]Thymidine incorporation. Inserts were removed and hepatocytes were washed twice with cold PBS. [*methyl*-^{3}H]Thymidine incorporated into cellular DNA was precipitated with 10% trichloroacetic acid (TCA) for 15 minutes at room temperature, the TCA was aspirated, and the hepatocytes were solubilized with a 0.3 M NaOH + 1% SDS solution for 10 minutes at room temperature. Lysates were neutralized with an equal volume of 0.3 M HCl and assayed for β-emission on a liquid scintillation counter (*S18*).

Statistics

Statistical significance was calculated using GraphPad Prism (GraphPad Software) by unpaired Student's *t* test for isolated pairs or by analysis of variance (one-way ANOVA, Bonferroni post-test) for multiple comparisons. Data are shown as the mean ± SEM.

Supplemental Figures

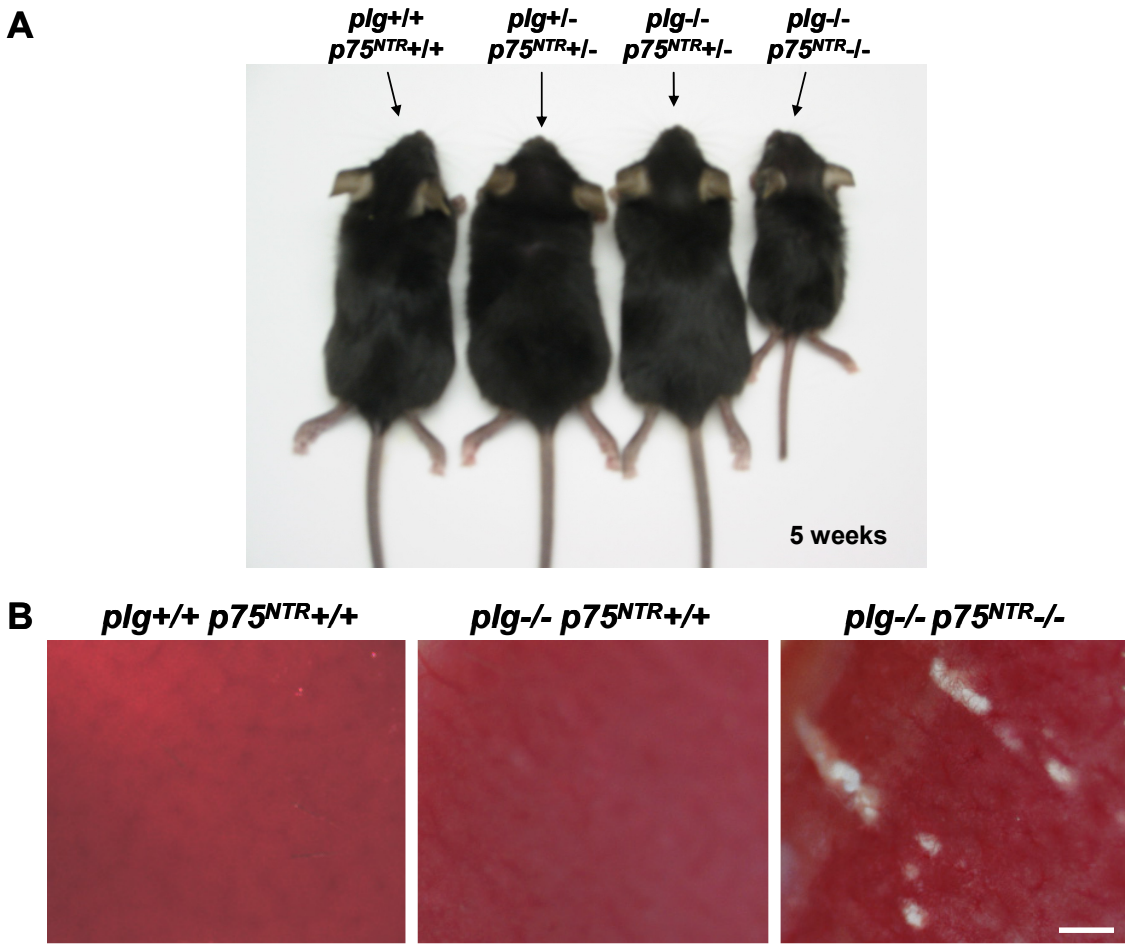

Figure S1. Genetic loss of p75^{NTR} exacerbates the effects of plasminogen deficiency. **(A)** Representative appearance of a *plg+/+p75^{NTR}+/+* mouse, a *plg+/-p75^{NTR}+/-* mouse, a *plg-/-p75^{NTR}+/-* mouse, and a *plg-/-p75^{NTR}-/-* mouse at 5 weeks of age. Heterozygous (+/-) mice are phenotypically undistinguishable from homozygous (+/+) mice. **(B)** Representative stereoscope images of the livers of 10 week old *plg+/+p75^{NTR}+/+* (left), *plg-/-p75^{NTR}+/+* (middle), and *plg-/-p75^{NTR}-/-* (right) mice. Scale bar, 0.4 mm.

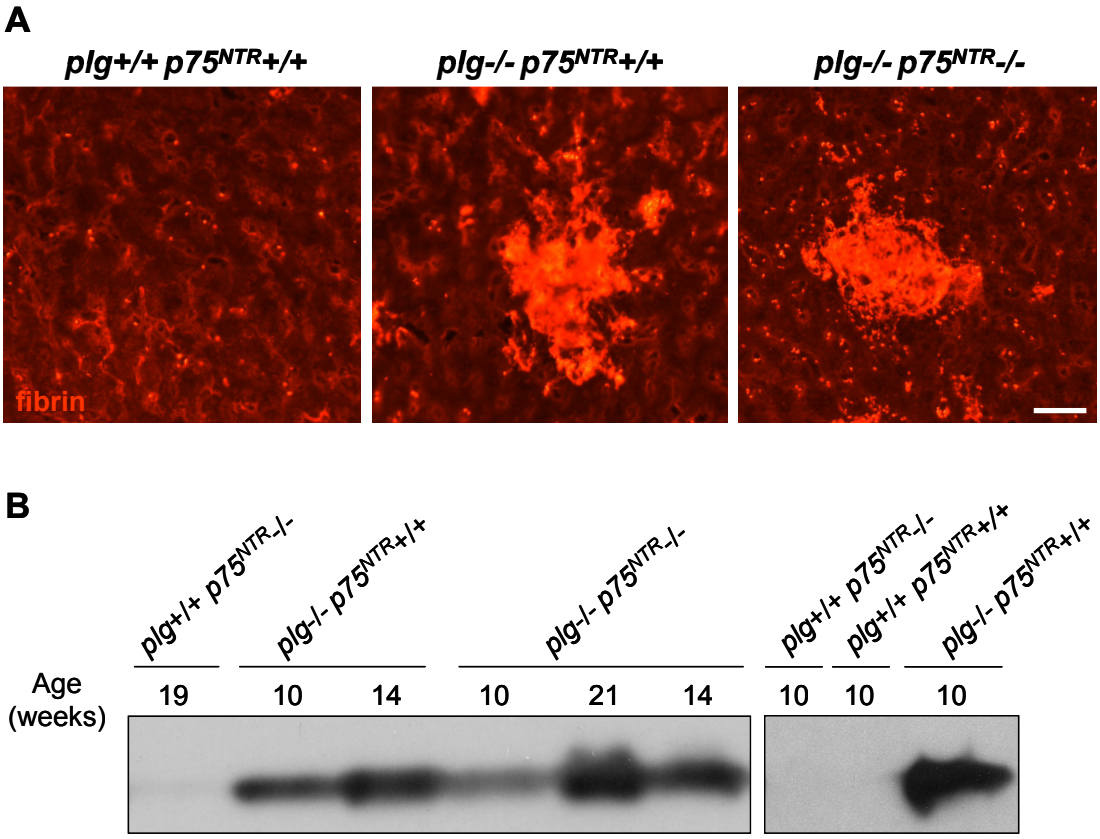

Figure S2. Genetic loss of p75^{NTR} does not change fibrin levels in the *plg-/-* mice.
(A) Immunochemical detection of fibrin(ogen) (red) in liver sections of 10 week old *plg*+/+*p75*^{NTR}+/+ (left), *plg-/-p75*^{NTR}+/+ (middle), and *plg-/-p75*^{NTR}-/- (right) mice. Normal mice (left) exhibit granular fibrin(ogen) staining indicative of blood vessel morphology. Both *plg-/-p75*^{NTR}+/+ (middle) and *plg-/-p75*^{NTR}-/- (right) liver contain large areas of fibrin deposits outside the vasculature. **(B)** Immunoblot detection of fibrin isolated from livers of *plg*+/+*p75*^{NTR}+/+, *plg*+/+*p75*^{NTR}-/-, *plg-/-p75*^{NTR}+/+, and *plg-/-p75*^{NTR}-/- mice, ages 10 to 21 weeks. Scale bar, 31 μm.

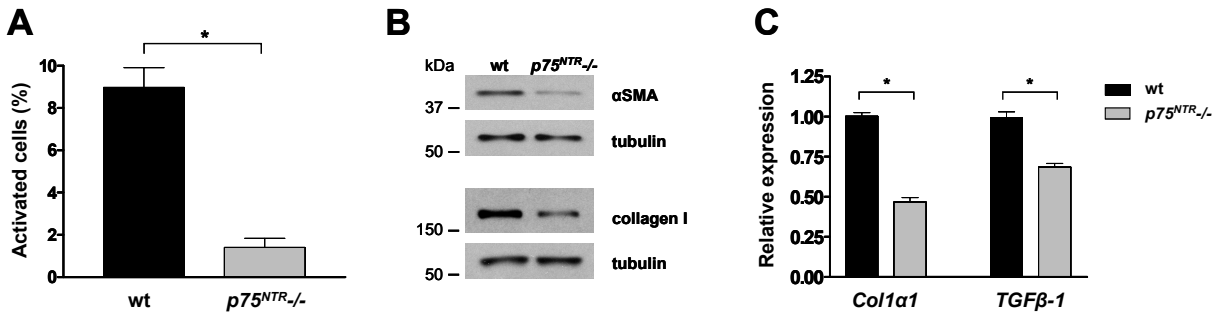

Figure S3. *p75^{NTR}-/-* HSCs fail to differentiate compared to wild-type HSCs.
(A) Quantification of HSC activation isolated from wild-type (wt) and *p75^{NTR}*-/- mice. Wt and *p75^{NTR}*-/- HSCs were cultured for 21 days. Activated HSCs were detected via αSMA immunostaining. Percentage of activated HSCs was quantified by counting the number of αSMA+ myofibroblasts per total number of nuclei per field. Experiments were performed four times in duplicates. Bar graph represents mean ± SEM. (**P*<0.0001; by unpaired Student's *t* test). **(B)** Western blot analysis of HSC protein extracts shows reduced expression of αSMA and collagen I in *p75^{NTR}*-/- HSCs when compared to control wt HSCs after 8 days in culture. **(C)** Real-time PCR analysis of gene expression of the myofibroblast markers *Col1α1* and *TGFβ-1* in wt and *p75^{NTR}*-/- HSCs after 14-21 days in culture. For each sample, gene expression data was normalized to HPRT gene expression and presented as fold change versus wt. Experiments were performed three times in duplicates. Graphs represent mean ± SEM (**P*<0.0001; by unpaired Student's *t* test).

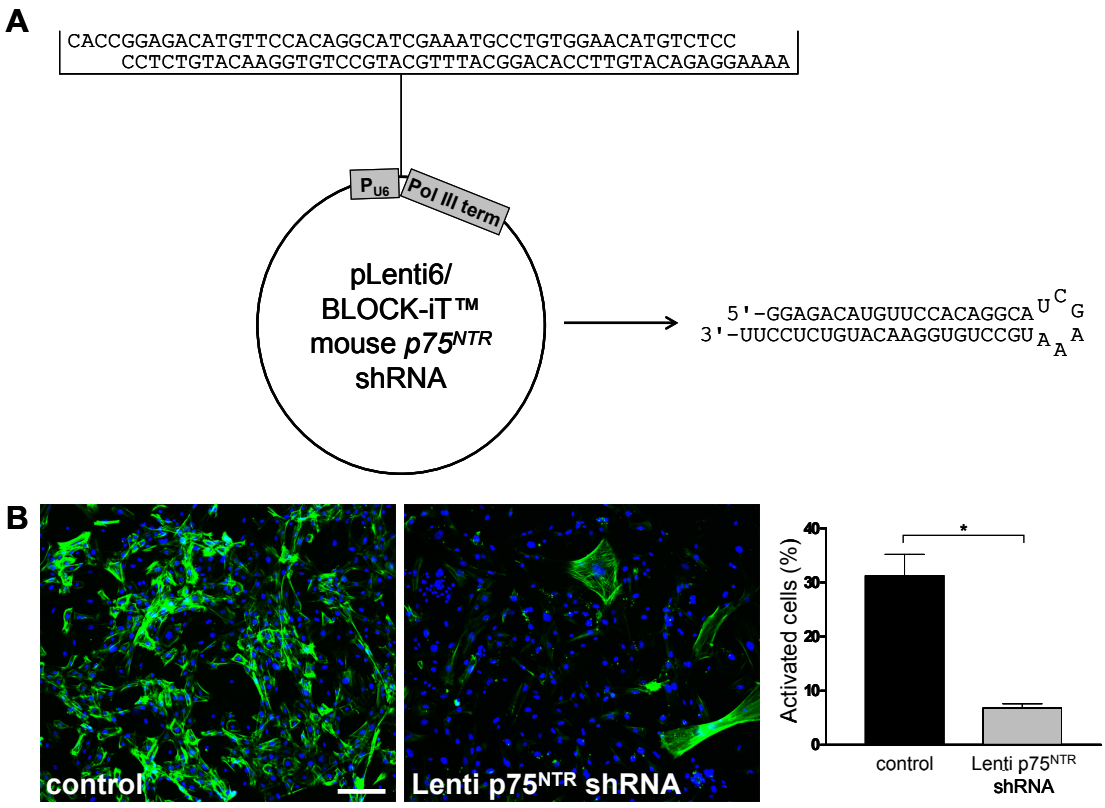

Figure S4. Lentiviral delivery of *p75*^{NTR} shRNA significantly decreases wild-type HSC activation.
(A) Vector map for the pLenti6/BLOCK-iT™ mouse *p75*^{NTR} shRNA vector. Nucleotide sequence shown at top was inserted into the pLenti6/BLOCK-iT™ vector. The predicted stem-loop structure of the mouse *p75*^{NTR} short hairpin RNA expressed from the vector is depicted at the right. **(B)** Wild-type (wt) HSCs transduced with mouse *p75*^{NTR} shRNA lentivirus exhibit greatly diminished activation. Freshly isolated wt HSCs were infected with lentivirus on day 2. αSMA immunostaining (green) was performed on day 10. Nuclei are stained with DAPI (blue). Percentage of activated HSCs was quantitated by counting the number of αSMA+ myofibroblasts per total number of nuclei per field. Experiments were performed three times in duplicates. Data are mean ± SEM (*P<0.009; by unpaired Student's t test). Scale bar, 123 μm.

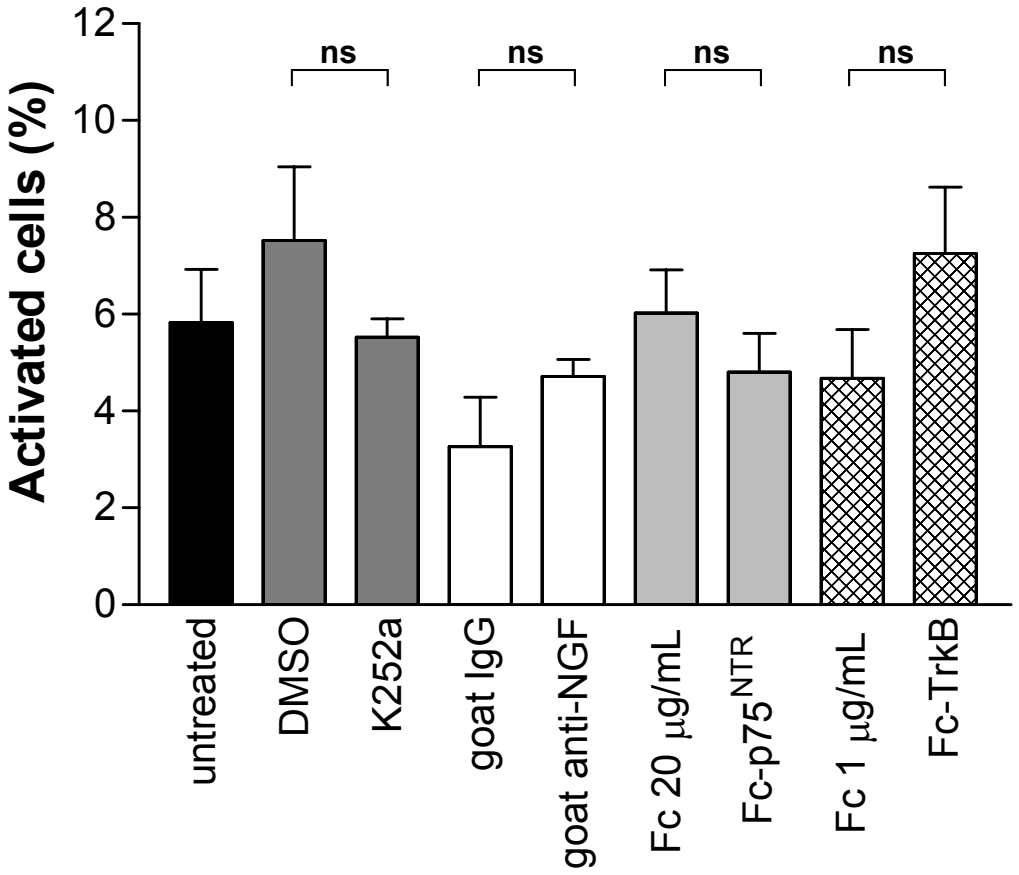

Figure S5. Trk inhibition or neurotrophin blocking have no effect on wild-type HSC differentiation.

Freshly isolated wild-type (wt) HSCs were treated on day 1 with the following: Trk inhibitor K252a (10 nM) or DMSO vehicle control; NGF-blocking antibody goat anti-NGF (2 μg/mL) or goat IgG control; neurotrophin scavenger Fc-p75^{NTR} or Fc fragment control (20 μg/mL); or BDNF scavenger Fc-TrkB or Fc fragment control (1 μg/mL). The medium was replaced with fresh medium containing treatments on days 3 and 5. αSMA immunostaining was performed on day 7 to assess activation. Percentage of activated HSCs was quantitated by counting the number of αSMA+ myofibroblasts per total number of nuclei per field. Experiments were performed three times in duplicates. Data are mean ± SEM (ns, not significant; by unpaired Student's *t* test).

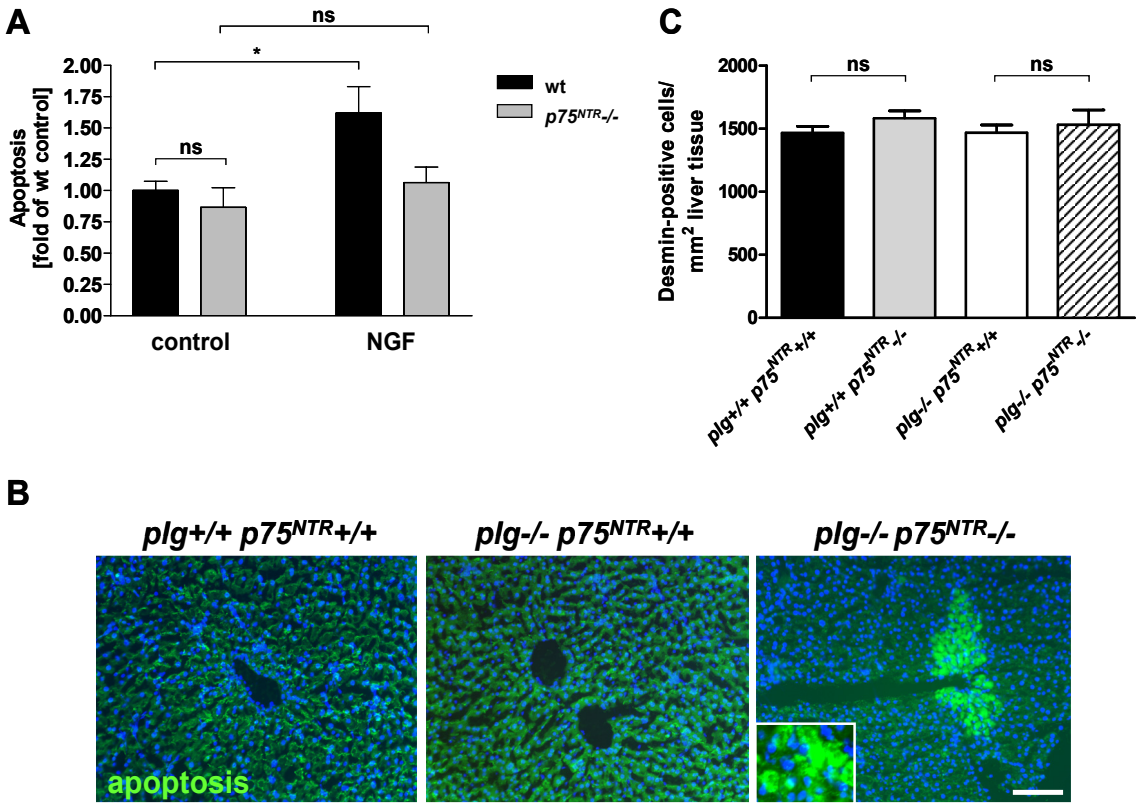

Figure S6. Genetic loss of p75^{NTR} does not affect apoptosis or developmental differentiation of HSCs.

(A) Apoptosis of HSCs in vitro. Wild-type (wt) and *p75^{NTR}*-/- HSCs (day 9-21) were incubated with or without 100 ng/mL NGF for 24 hours. Apoptosis, as measured by DNA fragmentation, was assayed by ELISA. Experiments were performed three times in duplicates. Data are mean ± SEM (*P<0.05; ns, not significant; by ANOVA). **(B)** Apoptosis of liver cells in vivo. Apoptosis, as measured by caspase activation, was assessed in liver sections of 10 week old *plg+/+p75^{NTR}+/+* (left), *plg-/-p75^{NTR}+/+* (middle), and *plg-/-p75^{NTR}-/-* (right) mice. Inset depicts apoptotic cells at a higher magnification, determined by morphology to be hepatocytes. **(C)** Quantitation of HSC number in vivo. HSCs were visualized in liver sections of 10 week old *plg+/+p75^{NTR}+/+*, *plg+/+p75^{NTR}-/-*, *plg-/-p75^{NTR}+/+*, and *plg-/-p75^{NTR}-/-* mice via immunostaining for desmin, a HSC marker. HSC number was determined by counting the number of desmin-positive cells per field. Data are mean ± SEM (ns, not significant; by unpaired Student's *t* test). Scale bars, 100 μm, (B); 35 μm, inset.

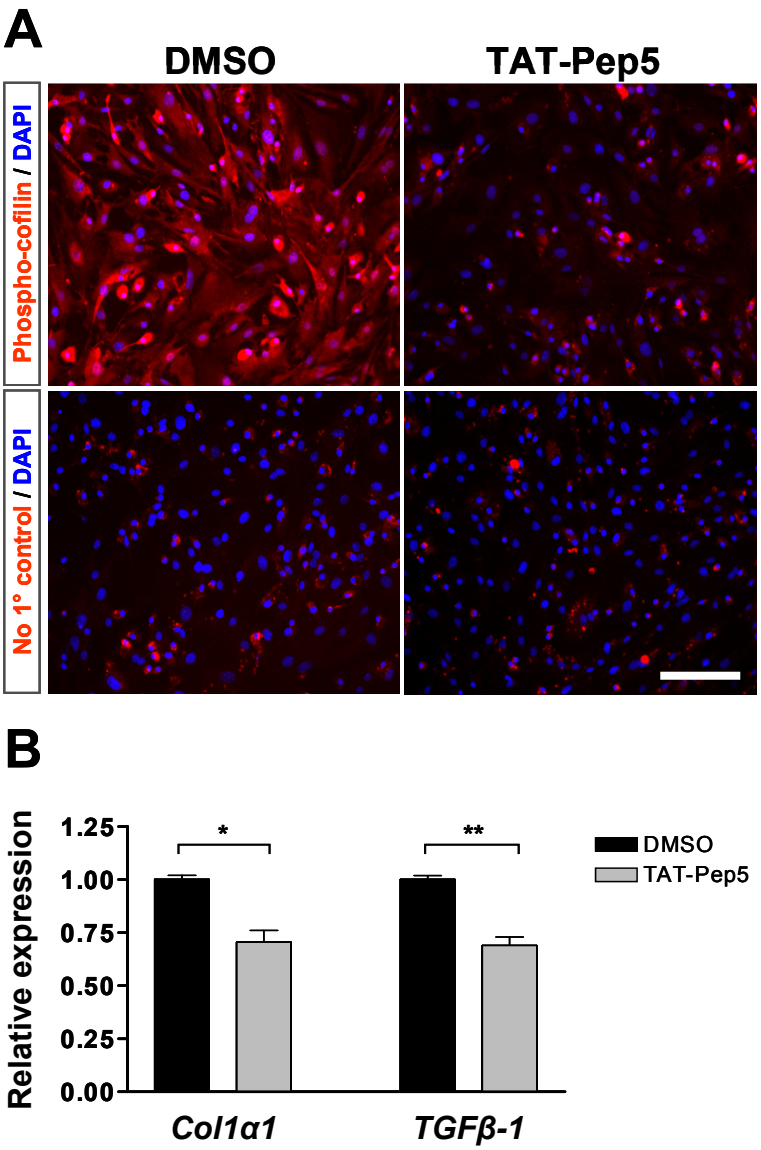

Figure S7. TAT-Pep5 treatment blocks p75^{NTR}-mediated Rho activation and diminishes expression of myofibroblast gene markers in wild-type HSCs.
(A) Examination of Rho activation in wild-type (wt) HSCs treated with TAT-Pep5. Freshly isolated wt HSCs were treated every day starting at day 1 with either TAT-Pep5, a specific inhibitor of p75^{NTR}-mediated Rho activation, or DMSO (vehicle) control. Phosphorylated cofilin, a marker indicative of Rho activation, was detected by immunostaining (red) on day 7 (top panels). Primary antibody omission served as an immunostaining control (bottom panels). Nuclei are stained with DAPI (blue). **(B)** Expression of myofibroblast markers in wt HSCs (day 7-21) treated with DMSO or TAT-Pep5. *Col1α1* and *TGFβ-1* gene expression levels were quantitated using real-time PCR. For each sample, gene expression data was normalized to HPRT gene expression and presented as fold change versus DMSO-treated control. Experiments were performed three times in duplicates. Graph represents mean ± SEM (*P<0.003; **P<0.001; by unpaired Student's t test). Scale bar, 175 μm.

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