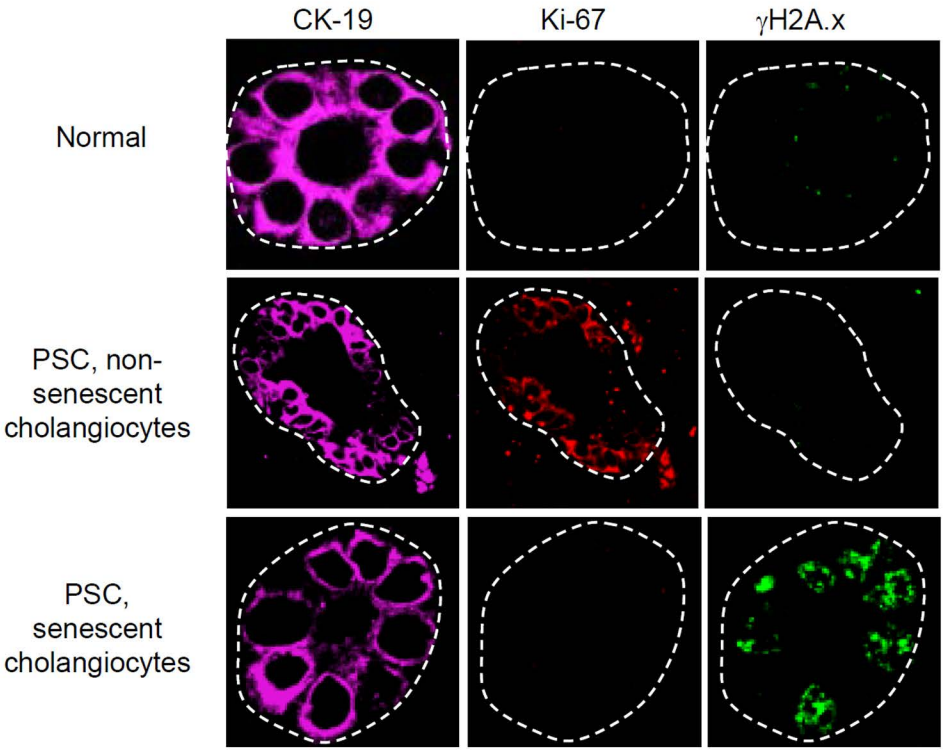
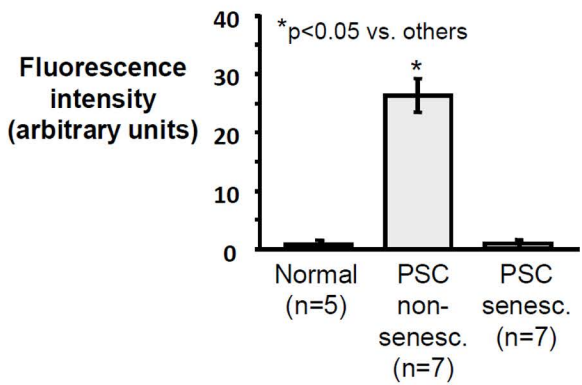
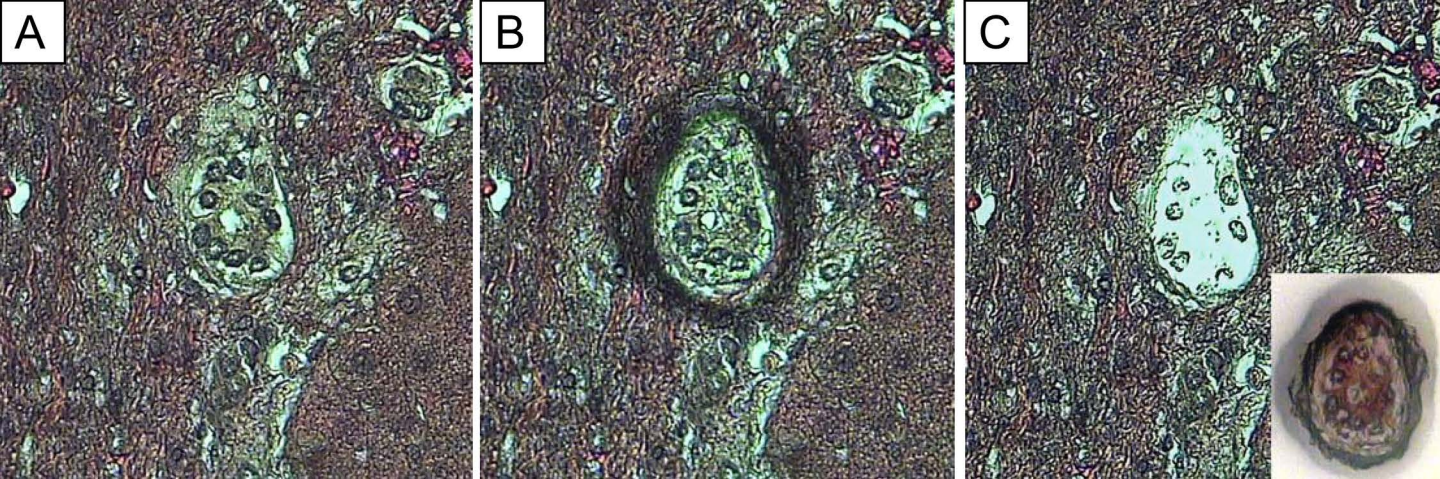
A.


CK-19
Ki-67
γH2A.x
Normal
PSC, non-senescent cholangiocytes
PSC, senescent cholangiocytes

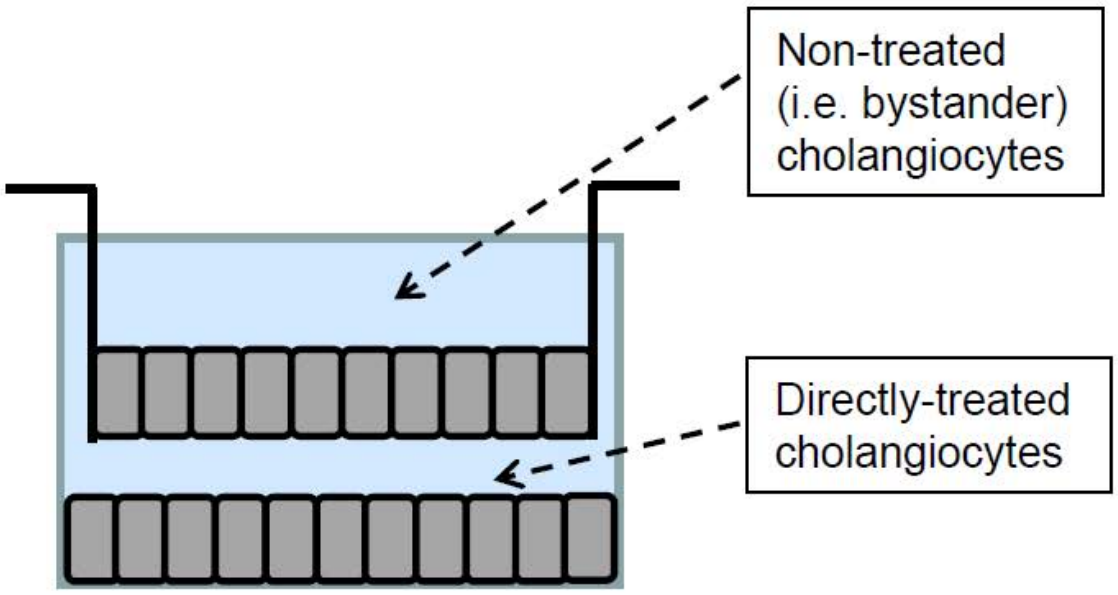
B.


Fluorescence intensity (arbitrary units)
40
30
20
10
0
*p<0.05 vs. others
*
Normal (n=5)
PSC non-senesc. (n=7)
PSC senesc. (n=7)

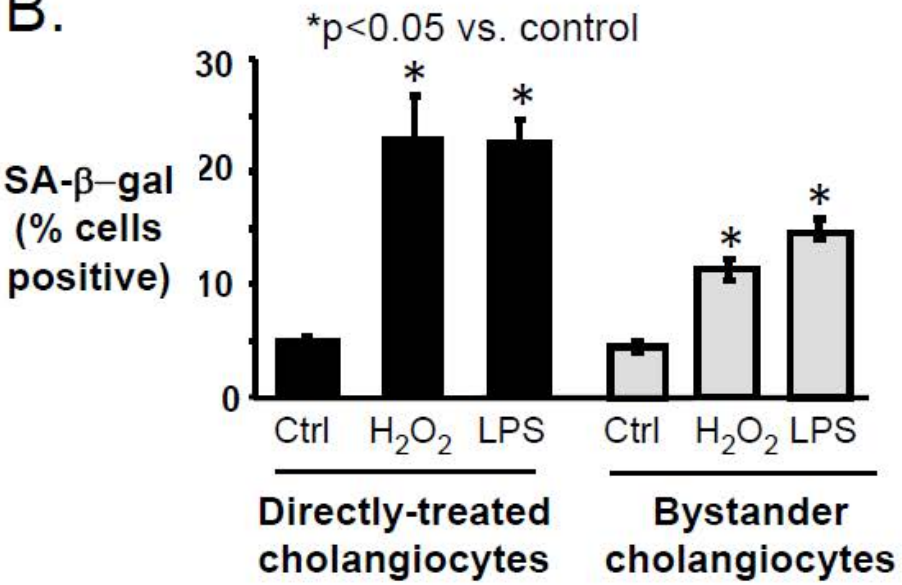

A
B
C

A.

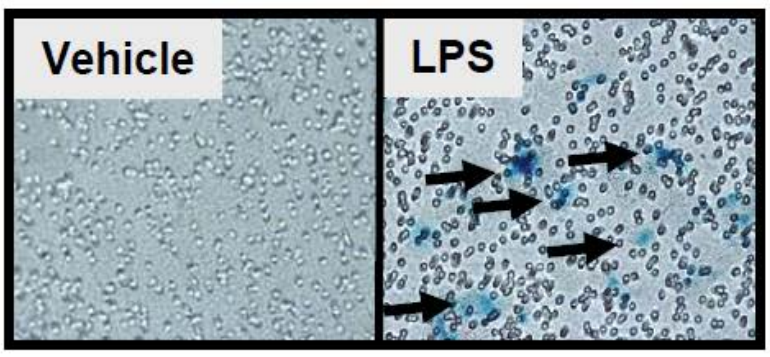
Schematic of cholangiocyte co-culture senescence model



B.



C.



SA-β-gal staining

SUPPLEMENTAL MATERIALS AND METHODS

Confocal immunofluorescence (IF) microscopy

Antibodies used for confocal IF microscopy included: cytokeratin (CK) 19 (goat polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA or mouse monoclonal IgG_1, Sigma-Aldrich, St. Louis, MO), γH2A.x (rabbit polyclonal IgG, AbCam, Cambridge, MA), Ki-67 (mouse monoclonal IgG_1, Santa Cruz Biotechnology), IL-6 (rabbit polyclonal IgG, Santa Cruz Biotechnology), IL-8 (rabbit polyclonal IgG, Novus Biologicals, Littleton, CO), plasminogen activator inhibitor-1 (PAI-1; rabbit polyclonal IgG, Santa Cruz Biotechnology), IL-10 (mouse monoclonal IgG_{2b}, Santa Cruz Biotechnology), chemokine (C-C motif) ligand 2 (CCL2; rabbit polyclonal, Abgent, San Diego, CA), N-Ras (rabbit polyclonal, Santa Cruz Biotechnology), or activated N-Ras (mouse monoclonal IgG_1, NewEast Biosciences, King of Prussia, PA) and corresponding fluorophore-conjugated anti- mouse, rabbit, or goat secondary antibodies (Invitrogen, Grand Island, NY). Coverslips were mounted using ProLong Gold antifade reagent with DAPI stain (Invitrogen).

Fluorescence in situ hybridization (FISH)

Telomere FISH was performed using PNA TelC-FITC telomere probe (PNA Bio, Thousand Oaks, CA) with tyramide signal amplification based on a combination of the manufacturer's protocol and previously described methods.(1,2) Liver sections were deparaffinized, rehydrated, boiled in citrate buffer for 20 minutes, prehybridized and heat denatured (85° for 10 minutes), and hybridized to TelC telomere probe diluted to 100 ng/ml in hybridization solution (70% formamide, 10mM Tris, pH 7.5, and 0.5% blocking reagent; PerkinElmer, Waltham, MA). Anti-fluorescein peroxidase-conjugated antibody (Rockland Immunochemicals, Gilbertsville, PA) was added to the slides at a 1:1000 dilution. Slides were washed in wash buffer (0.1M Tris-HCl pH 7.5, 0.15M NaCl, and 0.2% Triton X-100), incubated in a tyramide amplification solution (PerkinElmer), and washed again. Coverslips were mounted as described above. Fluorescent telomere probe signals were visualized using a Zeiss 510 LSM confocal microscope as previously described(2,3) and quantitated for each cholangiocyte nucleus, and this value was divided by the DAPI fluorescence signal for the corresponding nucleus, thus correcting for potential confounders

such as nuclear cutting planes and DNA content.(1,4) The resulting normalized (i.e. relative) telomere signal is linearly proportional to the mean telomere length as assessed independently by Southern blotting.(1)

Detection of p16 mRNA was performed as described previously.(5) Briefly, liver sections were deparaffinized, rehydrated, and boiled in sodium citrate buffer. Slides were prehybridized in a 4X SSC solution containing 3% BSA at 55°C. Slides were then incubated with either a scrambled non-specific probe or p16 LNA probe (Exiqon, Woburn, MA) diluted in hybridization buffer containing 10% dextran sulfate in 4X SSC. Slides were hybridized at 55°C for 1 hour and then submitted to a series of 5 washes of decreasing stringency. Coverslips were then mounted as described above.

N-Ras activation assay

N-Ras activation was assessed in NHC cells cultured in the presence or absence of 200 ng/ml of LPS (Invivogen) for 0, 1, 2, 6, and 10 days using an N-Ras activation kit (Cytoskeleton, Denver, CO) following the manufacturer's protocol. NHC cells were serum starved overnight prior to treatment with LPS. Cell media was removed at each time point and cells were washed twice on ice with 1X PBS. Cell lysis buffer (Cytoskeleton) containing protease inhibitors (Roche Diagnostics) was added to cells, cells were scraped in lysis buffer into microfuge tubes and centrifuged at 4ºC to remove cellular debris. Protein concentrations were determined, and samples were diluted accordingly with cold cell lysis buffer. Each sample was incubated with Raf-RBD beads for 1 hour with rotation at 4ºC. Protein-bound beads were twice centrifuged, washed in wash buffer (Cytoskeleton), resuspended and boiled in SDS sample buffer containing β-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to membrane, and blotted for presence of N-Ras using an anti-N-Ras mouse monoclonal antibody (Santa Cruz Biotechnology). Total Raf-RBD was used as loading control.

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5. de Planell-Saguer, M., Rodicio, M. C., and Mourelatos, Z. (2010) *Nat Protoc* **5**, 1061-1073

Supplementary Table 1. PCR primers

Transcript	Sense (5' to 3')	Antisense (5' to 3')
p16	CAGCAGCCGCTTCCTAGAAGAC	AGCCAGGTCCACGGGCAGAC
Ki-67	GCAACGACATGAAAACCAACAAAG	ACTTTGTCTCTAGGTATGGGTTTC
IL-6	CTCACCTCTTCAGAACGAATTGAC	ATCTGTTCTGGAGGTACTCTAGG
IL-8	CCTGATTTCTGCAGCTCTGTGTG	TTCTCCACAACCCTCTGCACCC
CCL2	GATCTCAGTGCAGAGGCTCG	TTTGCTTGTCCAGGTGGTCC

Key: IL-6, interleukin 6; IL-8, interleukin 8; CCL2, Chemokine (C-C motif) ligand 2 (also referred to as monocyte chemotactic protein-1 [MCP-1]).

Supplementary Figure Legends

Supplementary figure 1. gH2A.x-positive cholangiocytes in PSC liver do not express Ki-67 cell proliferation marker, consistent with cellular senescence. **A.** Representative confocal co-immunofluorescence images using antibodies against CK-19 (far red), gH2A.x (green), and Ki-67 (red) in PSC and normal liver tissue. gH2A.x-positive cholangiocytes in PSC liver are negative for the proliferation marker Ki-67, as are cholangiocytes in normal liver. **B.** Semi-quantitative analysis of fluorescence intensity demonstrates decreased Ki-67 in gH2A.x-positive cholangiocytes in PSC liver, further supporting their senescent state.

Supplementary figure 2. Cholangiocyte laser capture microdissection (LCM) was performed on normal (n=3) and PSC (n=3) liver. Sections (4 μm) were H&E stained and immediately transferred to the LCM instrument. Ducts and cholangiocytes were identified morphologically at 10x-100x magnification. **A)** Pre-LCM photomicrograph with duct in center. **B)** Photomicrograph demonstrating laser burn surrounding a duct. **C)** Post-LCM photomicrograph with duct of interest cut out (inset: LCM cap with duct of interest).

Supplementary figure 3. Persistent treatment of cultured NHCs with LPS induces changes in mRNA expression consistent with cellular senescence. **A.** *In vitro* model of stress-induced senescence, wherein NHCs were exposed to LPS (or other) treatment and the induction of senescence was assessed over the course of 10 days. **B.** Using this model, we found that even with lower doses of LPS, cholangiocytes transition to a senescent state (based on SA-β-gal positivity), albeit at a slower rate as compared to higher doses, thus indicating time- and LPS dose- dependence in this process. **C.** Ki-67 expression, a marker of cell proliferation, is increased acutely but then returns to normal levels (i.e. that of untreated control cells) by 10 days. This suggests an initial replicative response to LPS as we have previously shown (O'Hara et al. J Biol Chem 2011, PMID 21757746). However, with persistent LPS treatment, there is diminution of the replicative response which is contemporaneous with the induction of cellular senescence during this interval.

Supplementary figure 4. Co-culture system shows LPS directly-induced senescent cholangiocytes promote senescence in bystander cholangiocytes. **A.** Cultured NHCs were treated with LPS (or positive control H_2O_2) for 10 days, when >30% are senescent. Cells were washed repeatedly and cultured for another 48 hours. Following another change in media, bystander cholangiocytes, grown on 0.2 mm filter inserts, were placed in the wells containing the LPS directly-induced senescent cells. **B.** Quantitation of senescence in the directly treated and bystander cells. **C.** Bystander cells stained for SA-b- gal and visualized by bright field microscopy. Few SA-b- gal positive bystander cells were observed in vehicle treated wells, while numerous positive bystander cells were observed when cultured in the presence of senescent cholangiocytes.