Supplementary Information for

Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its etiology

Ingmar Mederacke, Christine C. Hsu, Juliane S. Troeger, Peter Huebener, Xueru Mu, Dianne H. Dapito, Jean-Philippe Pradere, Robert F. Schwabe*

* Corresponding author. E-mail: rfs2102@cumc.columbia.edu

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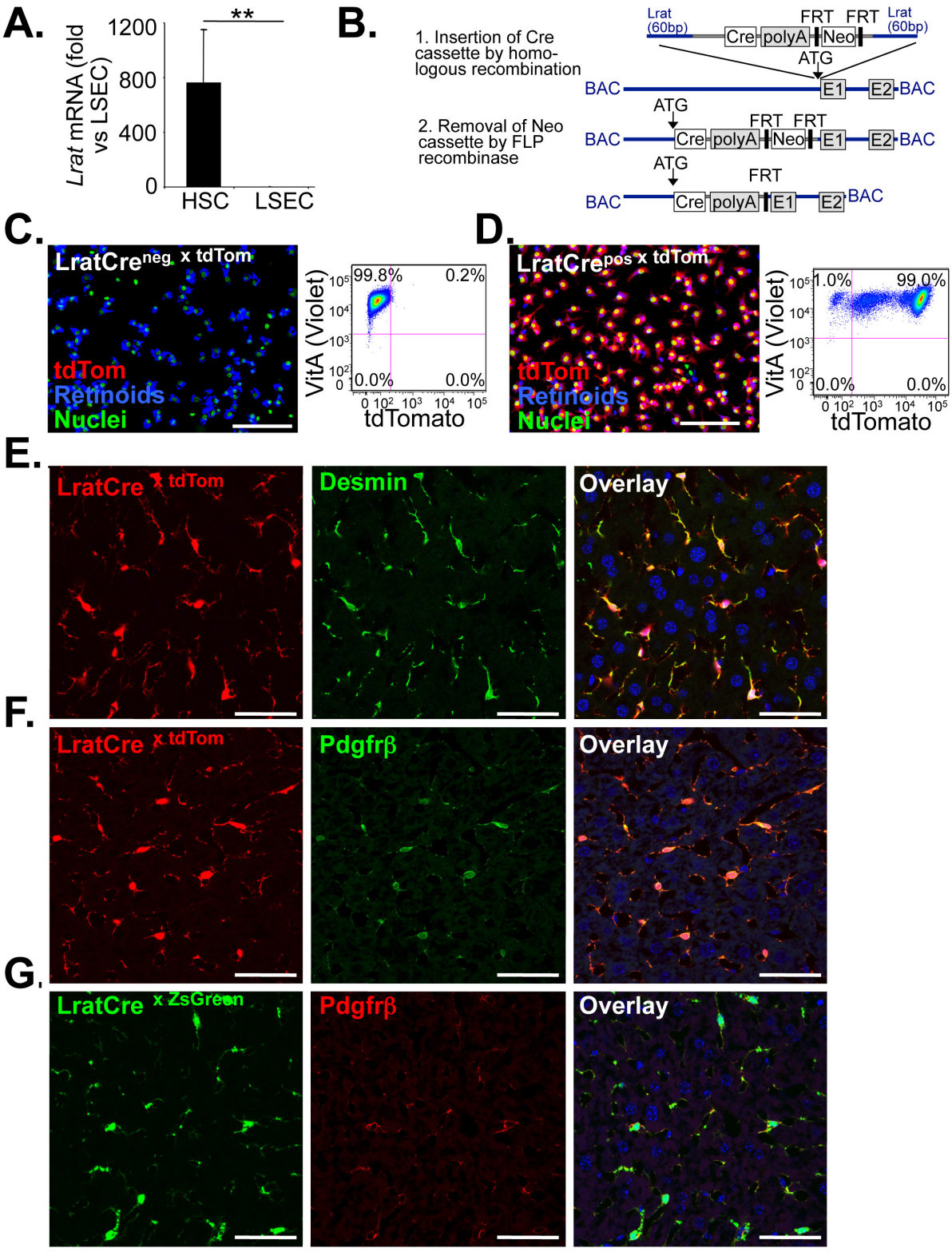
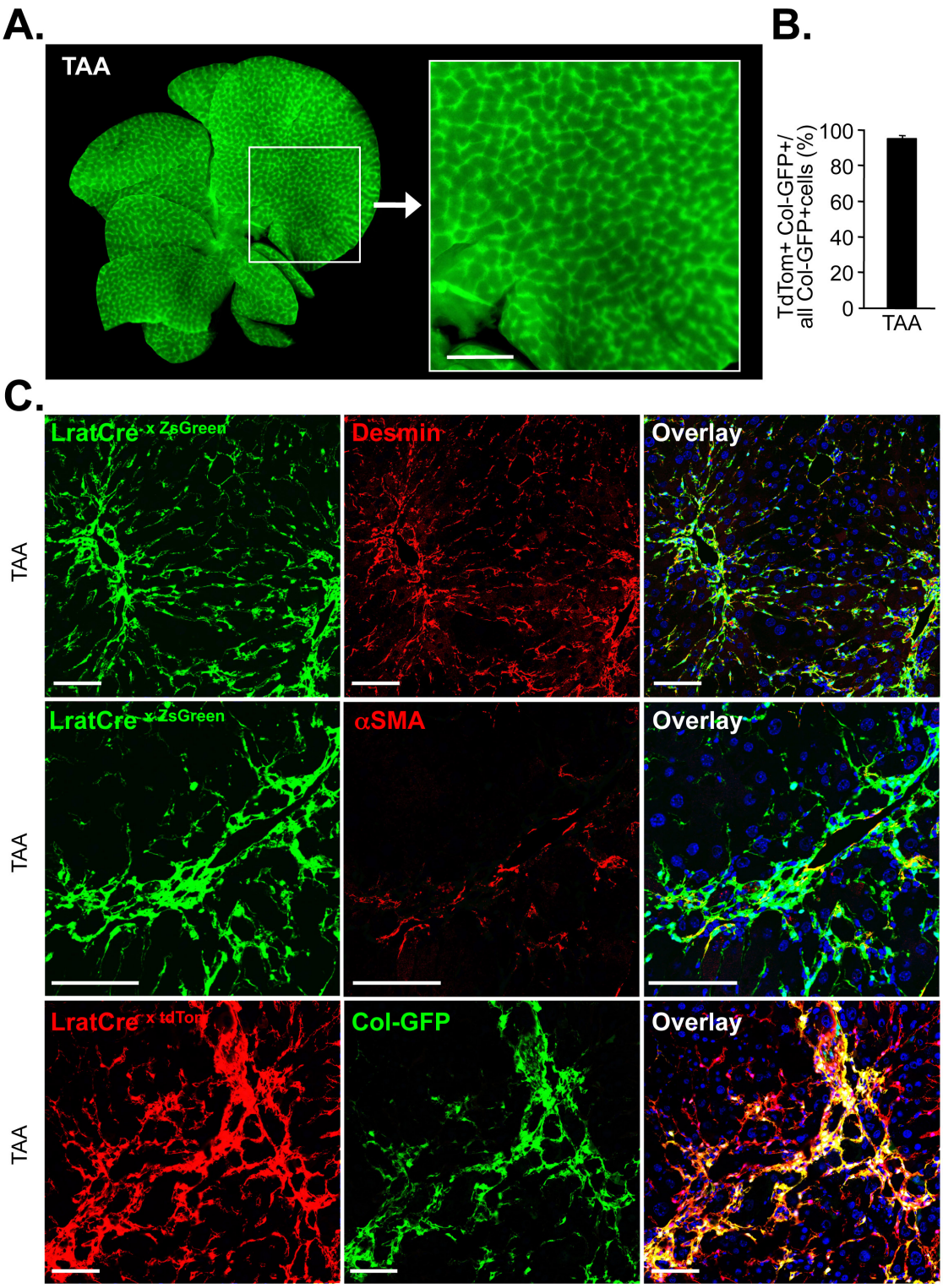
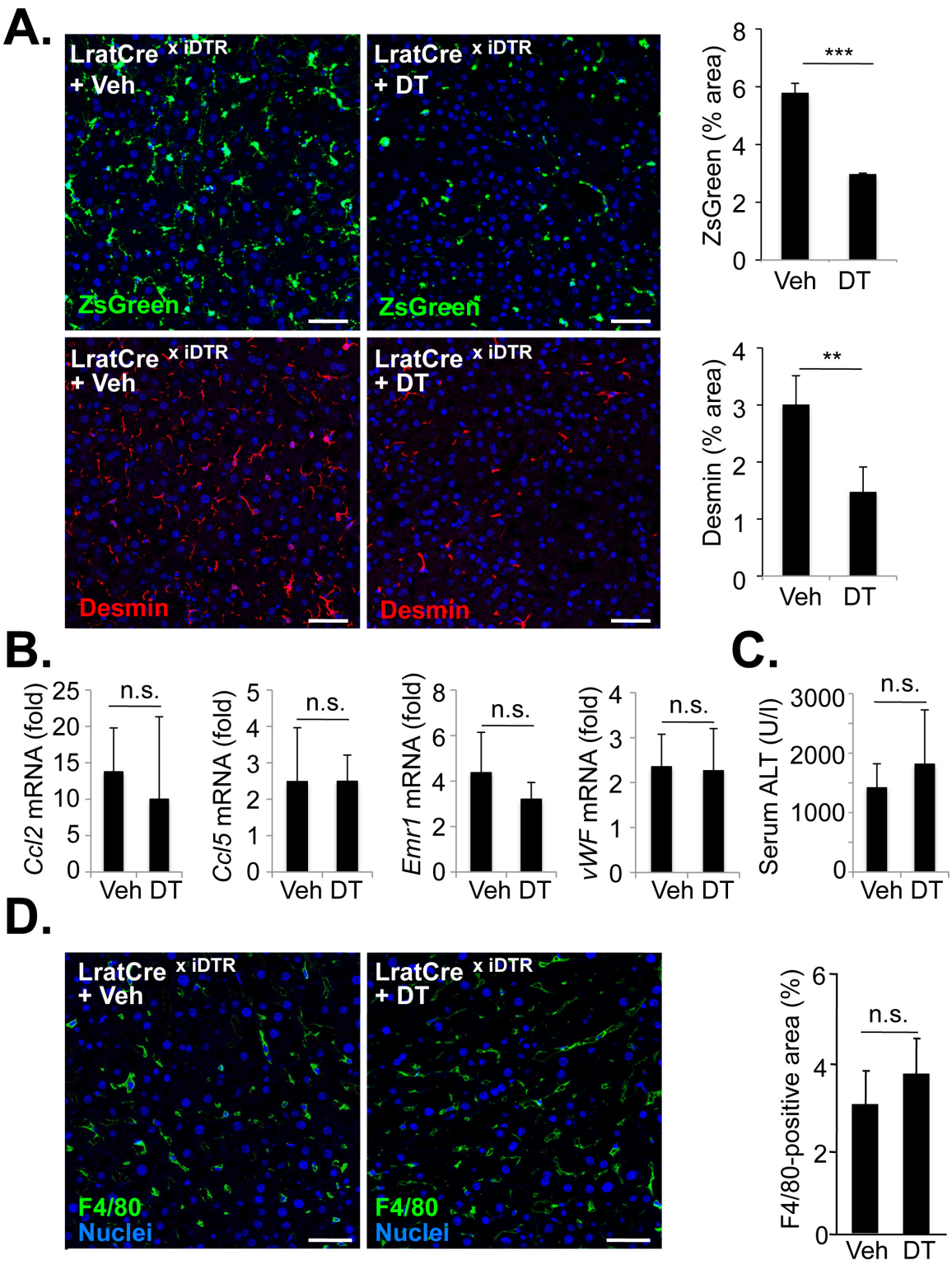


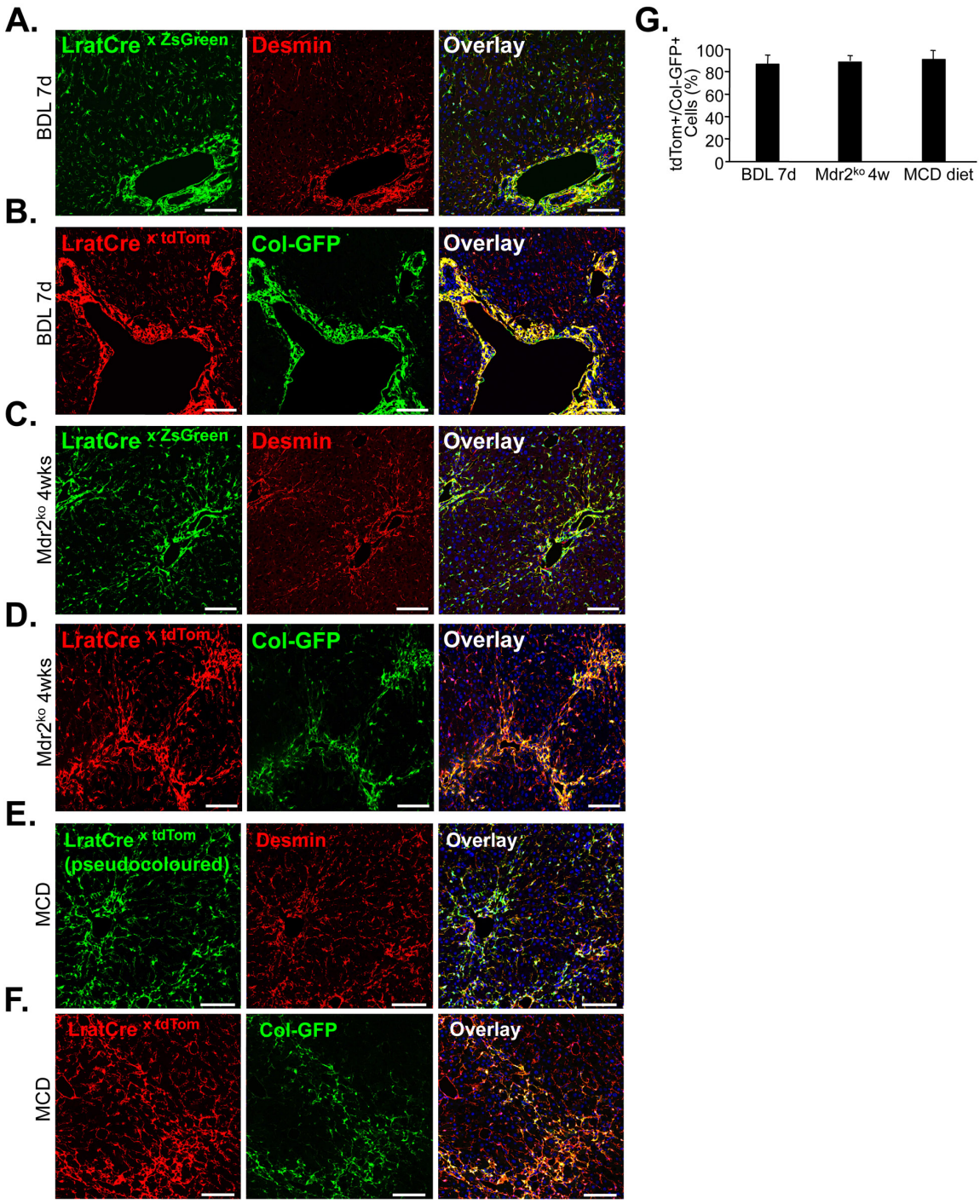
Supplementary Figure S1. Characterization of LratCre-mediated recombination in the liver. A. Expression of *Lrat* mRNA was compared between primary HSC and primary LSEC, isolated from 18 week old mice, using qPCR (n=3 per group). **B.** Displayed is the LratCre construct employed for generation of BAC-transgenic mice. **C-D.** HSCs were isolated from LratCre negative (C) and LratCre-positive mice (D) expressing tdTomato Cre reporter and either plated for 24h or analyzed by flow cytometry. **E-G.** Colocalization of LratCre-induced tdTomato or ZsGreen with desmin or Pdgfrβ was determined by immunohistochemical staining and confocal microscopy. Scale bars 100 μm (C-D), 50 μm (E-G). Data are shown as means ± SD. **p<0.01 (determined by Student t-test).



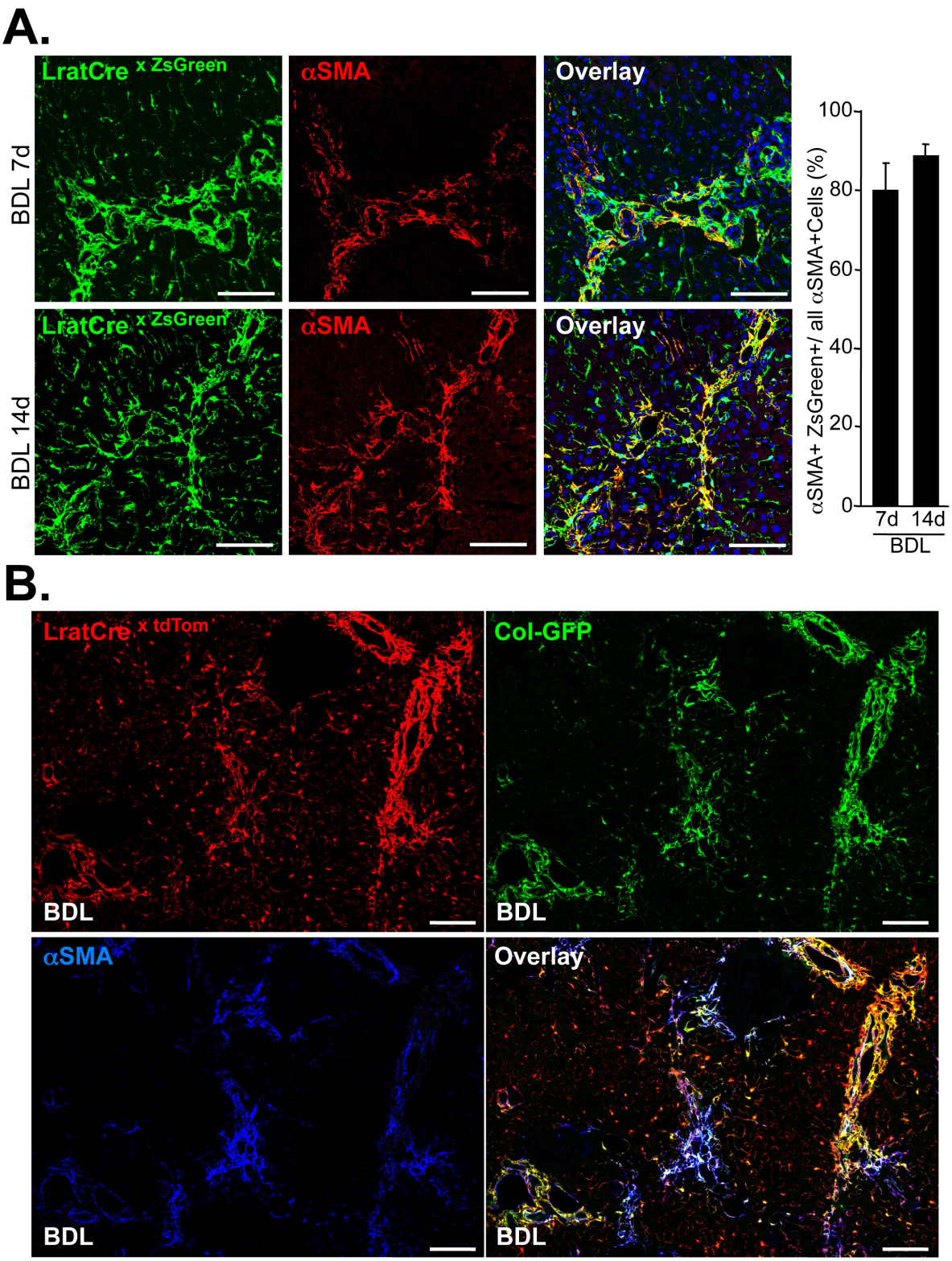
Supplementary Figure S2. Hepatic stellate cells are the principal source of myofibroblasts in thioacetamide-induced liver fibrosis. A. Representative fluorescent images of whole livers from six weeks thioacetamide (TAA)-treated (n=4) LratCre-positive mice, expressing ZsGreen Cre reporter, show macroscopic fibrotic septa. **B.** Quantification of Col-GFP-expressing cells that are derived from LratCre-labeled tdTomato-positive HSCs in fibrosis induced by six weeks TAA treatment (n=3). **C.** Frozen liver sections from TAA-treated LratCre-positive mice (n=4) were stained with desmin (upper panel) or αSMA (middle panel) to demonstrate co-localization of HSC marker desmin or αSMA and LratCre-induced ZsGreen by confocal microscopy. Confocal microscopy was employed to show co-localization of Col-GFP reporter, marking activated myofibroblasts, and LratCre-induced tdTomato expression (lower panel) after six week TAA treatment (n=3). Scale bars 1 mm (A), 100 μm (C). Data are shown as means ± SD.



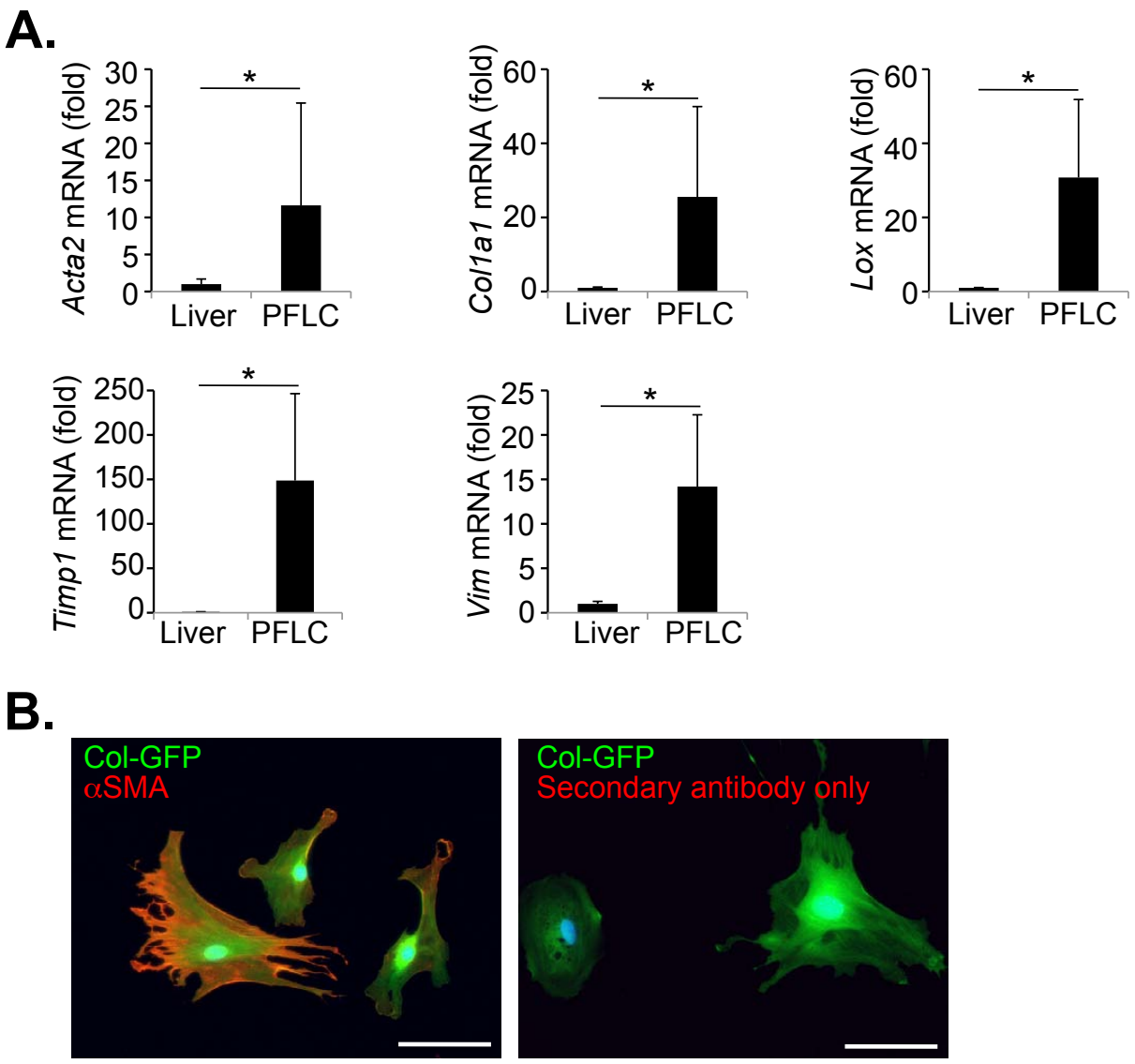
Supplementary Figure S3. HSC depletion via LratCre-induced iDTR expression. A. Depletion of HSCs in normal livers was investigated in LratCre-positive mice expressing Cre-inducible diphteria toxin receptor (iDTR) followed by treatment with diphteria toxin (DT, n=3) or vehicle (n=4). Depletion of HSCs was assessed by ZsGreen and Desmin expression. **B-D.** LratCre-positive mice expressing Cre-inducible diphteria toxin receptor (iDTR) were treated with 4 injections of CCl_4 and 4 injection of DT (n=4) or vehicle (n=4). Expression of inflammatory genes and endothelial cell marker *vWf* was determined by qPCR (B). Liver injury was assessed by serum ALT levels (C). The number of F4/80-expressing liver macrophages was determined by immunohistochemistry (D). Scale bars 50 μm (A,D). Data are shown as means ± SD. n.s., non-significant. **p<0.01, ***p<0.001 (determined by Student t-test).



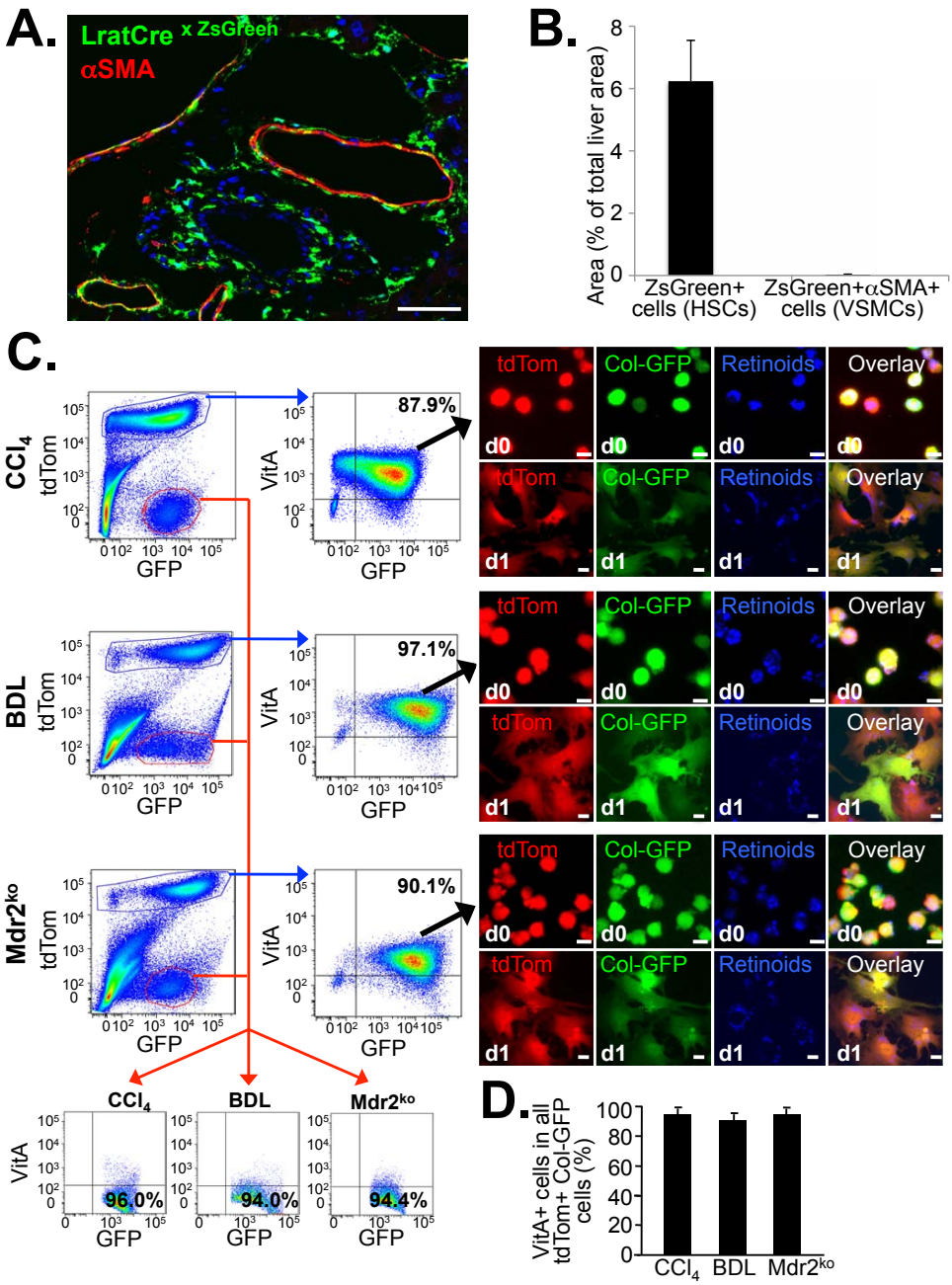
Supplementary Figure 4. LratCre-marked HSCs are the primary collagen-producing cells in early stages of cholestatic and in non-alcoholic steatohepatitis induced liver fibrosis. A. Mice expressing LratCre and ZsGreen Cre reporter underwent BDL for 7 days (n=1), followed by staining for HSC marker desmin. **B.** Mice expressing LratCre, tdTomato Cre reporter and Col-GFP underwent BDL for 7 days (n=4), and were analyzed by confocal microscopy**. C.** Mdr2ko mice expressing LratCre and ZsGreen were sacrificed at the age of 4 to 6 weeks (n=5) and stained for HSC marker desmin. **D.** Mdr2ko mice expressing LratCre, tdTomato Cre reporter and Col-GFP were analyzed at the age of 4 weeks (n=5) for co-localization of tdTomato and Col-GFP in the liver using confocal microscopy. **E-F.** LratCre mice expressing tdTomato Cre reporter and Col-GFP reporter (n=2) were treated with MCD diet for up to 9 weeks. Co-localization of desmin with tdTomato (E), and co-localization of tdTomato and Col-GFP (F) was demonstrated by confocal microscopy. **G.** Quantification of Col-GFP-expressing cells that are derived from LratCre-labeled tdTomato-positive HSCs after 7 day bile duct ligation (n=4), in 4 week old Mdr2ko mice (n=5) and after MCD diet (n=2). Scale bars 100 μm (A-F). Data are shown as means ± SD.



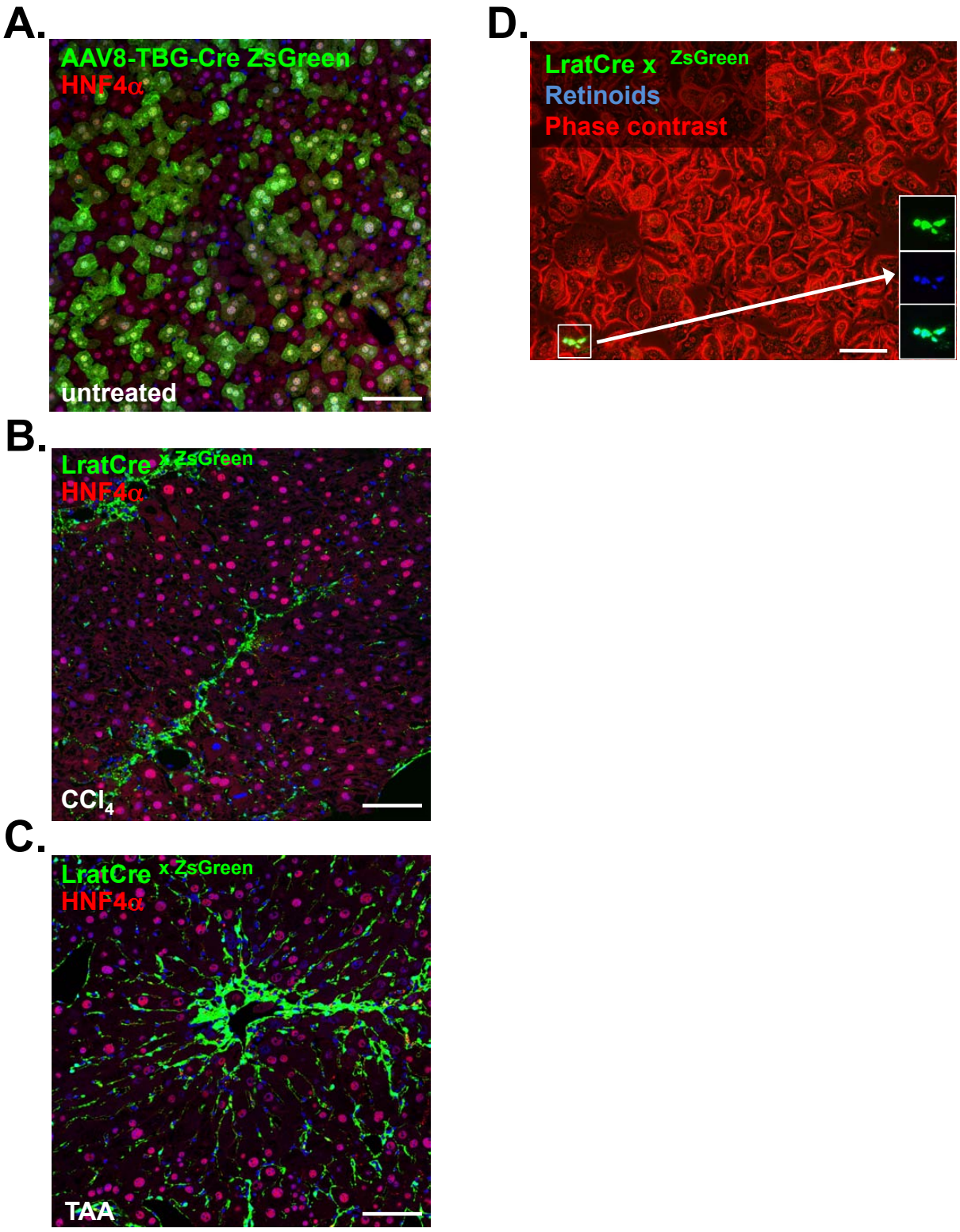
Supplementary Figure S5. LratCre marks αSMA-expressing myofibroblasts in liver fibrosis induced by bile duct ligation. A. Liver sections of 7 day bile duct-ligated (n=4) and 14 day bile duct-ligated (n=4) mice mice expressing LratCre and ZsGreen or tdTomato Cre reporter were stained for αSMA followed by confocal microscopy, to demonstrate and quantify co-localization of αSMA with LratCre-induced reporter expression. **B.** Co-localization of αSMA with tdTomato and Col-GFP in 14 day BDL-induced liver fibrosis was determined by confocal microscopy employing far-red secondary antibody for αSMA detection. Scale bars 100 μm (A-B). Data are shown as means ± SD.



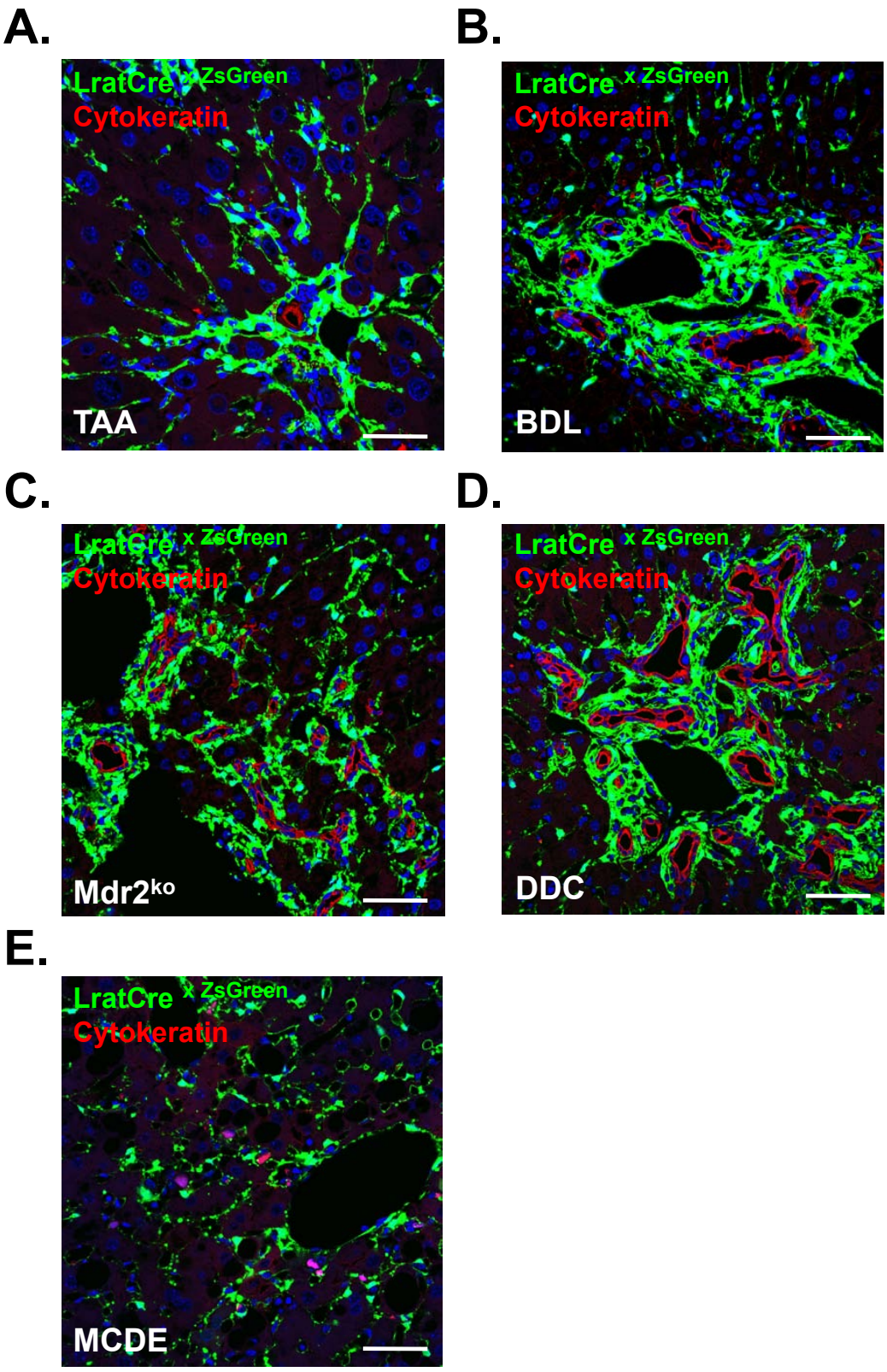
Supplementary Figure S6. Portal fibroblast-like cells have a gene expression characteristic for fibroblasts. A-B. Col-GFP-positive, tdTomato negative portal fibroblast-like cells (PFLC) were isolated by FACS as described. Gene expression of PFLC (n=5) was compared to whole liver (n=4) (A). αSMA expression was determined by immunofluorescent staining in PFLC cultured for 6 days. Staining without primary antibody confirmed specificity of the αSMA signal. Scale bar 50 μm. Data are shown as means ± SD. *p<0.05 (determined by Student t-test).



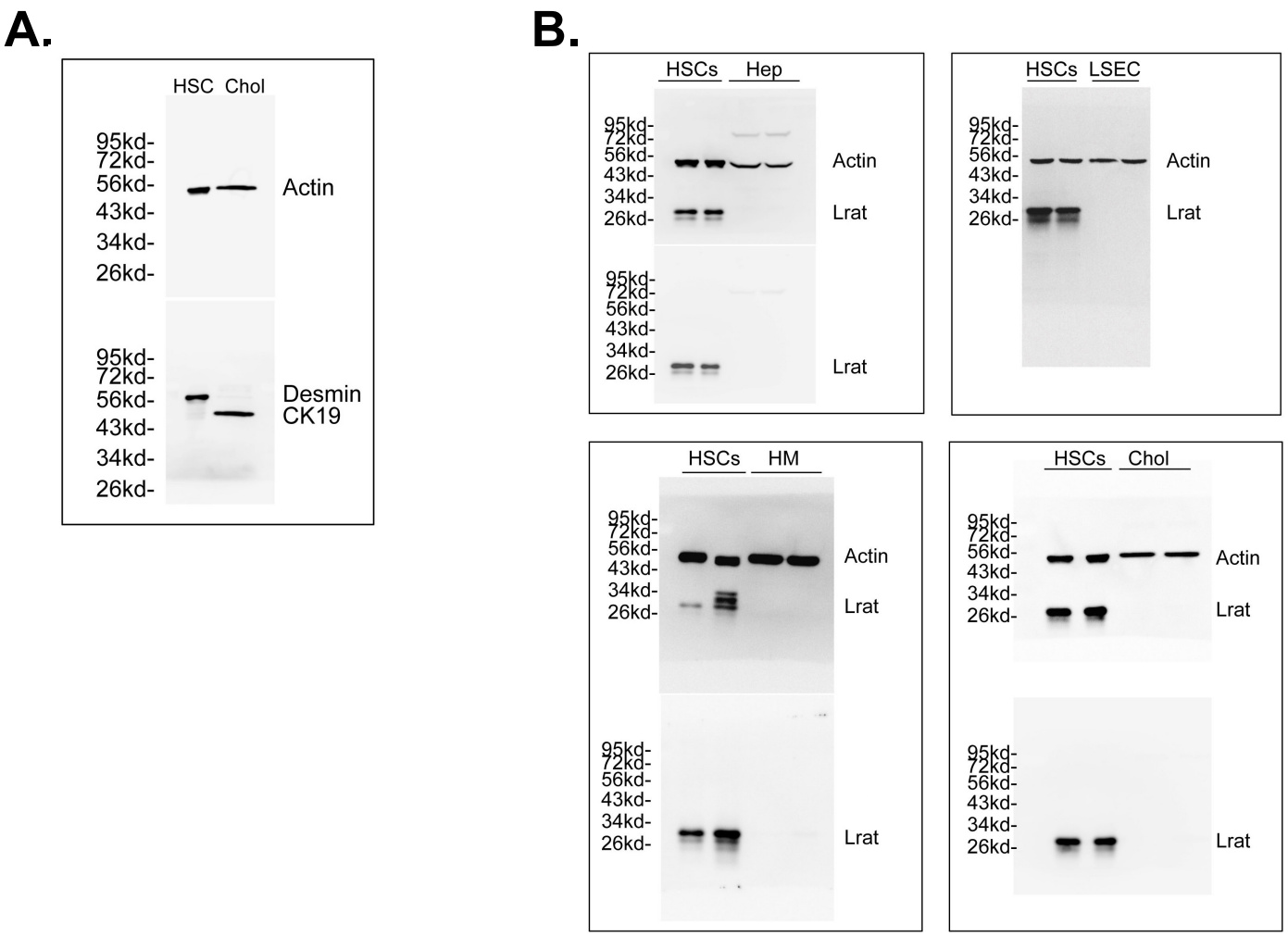
Supplementary Figure S7. Retinoid-positive LratCre-labeled HSCs are the primary collagen-producing cells in liver fibrosis. A-B. αSMA staining was employed to identify VSMCs, some of which are labeled by LratCre-induced ZsGreen (A). The number of ZsGreen-positive HSCs and ZsGreen-and aSMA-double positive VSMCs was determined by confocal microscopy of normal liver from LratCre-positive ZsGreen expressing livers (n=5) stained with αSMA, and quantified as percent area. **C.** Vitamin A fluorescence was employed to determine whether Col-GFP, tdTomato-double positive cells from CCl_4-treated, BDL and Mdr2ko mice were of HSC origin. Using flow cytometry, Col-GFP, tdTomato-double positive cells were gated (blue gates), followed by determination of vitamin A fluorescence in this cell populations (blue arrow). Sorted cells were additionally plated and analyzed by fluorescent microscopy to demonstrate presence of tdTom, Col-GFP and retinoids in each cell. Shown are freshly plated cells ("d0") and cells after 24h of culture ("d1") showing typical HSC morphology. To confirm the set vitamin A threshold in the FACS analysis, the Col-GFP positive, tdTomato-negative non-HSC population was gated (red gates) and analyzed for vitamin A fluorescence at the same settings (red arrows). **D.** The percentage of vitamin A containing HSCs among all Col-GFP, tdTomato-positive myofibroblasts was determined based on above flow cytometric analysis, and calculated for CCl_4-treated mice (n=5 isolations), BDL mice (n=4 isolations) and Mdr2ko mice (n=6 isolations). Scale bar 50 μm (A), 10 μm (C). Data are shown as means ± SD.

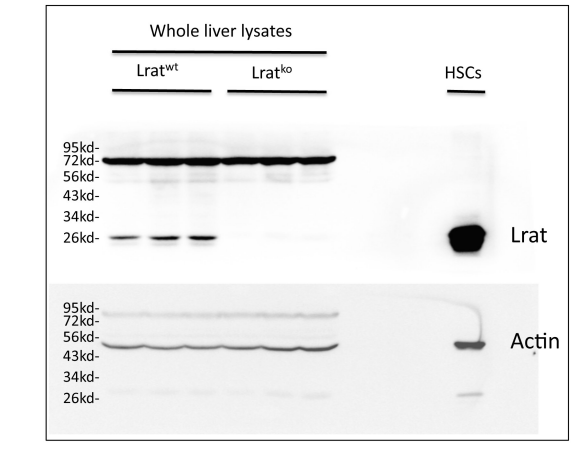


Supplementary Figure S8. LratCre-labeled HSCs do not contribute to hepatocyte generation in the CCl_4, TAA or DDC models of liver injury. A. As a positive control for ZsGreen-positive hepatocytes, mice were injected with low-dose AAV8-TBG-Cre and sacrificed one week later (n=1), followed by HNF4α immunohistochemistry and confocal microscopy. **B-C.** Liver sections from CCl_4-treated mice (n=4) (B) and TAA-treated mice (n=4) (C) expressing LratCre and ZsGreen Cre reporter were stained for HNF4α and analyzed by confocal microscopy. **D.** Image of primary hepatocytes from mice that received DDC diet for four weeks (n=1), followed by three weeks recovery, showing no ZsGreen-positive hepatocytes, and rare ZsGreen-positive HSCs, identified by their characteristic fluorescent retinoid-containing lipid droplets. Scale bars 100 μm.



Supplementary Figure S9. LratCre-labeled HSCs are not precursors of liver progenitor cells. Liver sections of TAA-treated (n=4) (A), 14 day bile duct-ligated (n=3) (B), Mdr2ko mice (n=4) (C), DDC-treated (n=3) (D) and MCDE-treated mice (n=4) (E) all expressing LratCre and ZsGreen Cre reporter, were stained for cytokeratin. Confocal microscopy revealed no co-localization of the liver progenitor marker cytokeratin with LratCre-induced ZsGreen expression. Scale bars 50 μm.





Supplementary Figure S10. A-B. Full size Western Blot for Figures 1F (A) and Figure 2A (B). **C.** Specificity of the Lrat antibody was determined using liver extracts from 3 wild-type and 3 Lrat knockout mice. Hepatic stellate cell extracts were used as additional positive control.