Cholangiocytes act as facultative liver stem cells during impaired hepatocyte regeneration

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After liver injury, regeneration occurs through self-replication of hepatocytes. In severe liver injury, hepatocyte proliferation is impaired—a feature of human chronic liver disease1,2. It is unclear whether other liver cell types can regenerate hepatocytes3-5. Here we use two independent systems to impair hepatocyte proliferation during liver injury to evaluate the contribution of non-hepatocytes to parenchymal regeneration. First, loss of β1-integrin in hepatocytes with liver injury triggered a ductular reaction of cholangiocyte origin, with approximately 25% of hepatocytes being derived from a non-hepatocyte origin. Second, cholangiocytes were lineage traced with concurrent inhibition of hepatocyte proliferation by β1-integrin knockout or p21 overexpression, resulting in the significant emergence of cholangiocyte-derived hepatocytes. We describe a model of combined liver injury and inhibition of hepatocyte proliferation that causes physiologically significant levels of regeneration of functional hepatocytes from biliary cells.

The identification of a liver stem or progenitor cell remains elusive6. Transplanted biliary progenitors can partly repopulate the liver’s hepatocyte population7,8 but it is still unproven whether endogenous biliary cells can regenerate hepatocytes9-11. Severe liver injury in human and animals impairs hepatocyte proliferation and causes ductular reactions to emerge12,13. Ductular reactions may arise from the biliary epithelium11,12 or through de-differentiation of hepatocytes13. Biliary cells in zebrafish models have been shown to regenerate the liver after massive hepatocyte loss14,15; however, this remains unproven in mammalian systems3,4,14. Lineage tracing of biliary ductular reactions into new hepatocytes, in the mouse, is dependent on both the severity and type of liver injury model, which have shown an inconsistent and limited contribution to hepatocyte regeneration3,4,15,16. Mouse lineage-tracing experiments do not typically recapitulate human disease, as significant hepatocyte replication is seen alongside ductular reactions. We therefore sought to develop a model in which ductular reactions occur on the background of impaired hepatocellular regeneration and to evaluate the regenerative capacity of the ductular reactions.

We lineage traced hepatocytes using the highly efficient adenovirus-associated viral AAV8.TBG.Cre injected into a R26RSTOPtdTomato mouse, thereby activating tdTomato (tdTom) expression in over 99.5% of hepatocytes (Extended Data Fig. 1a, b). These mice had either wild-type (WT) Irgb1 (β1-integrin11,15) or mutated Irgb1 (β1-integrin16) (Extended Data Fig. 1c-e), previously shown to eliminate β1-integrin expression17, thereby inhibiting hepatocyte growth factor signalling and impairing regeneration18.

AAV8.TBG.Cre-mediated β1-integrin ablation caused parenchymal damage with raised serum markers and cytoplasmic localization of high-mobility group box 1 protein (HMGB1), a damage-associated molecular pattern (DAMP) associated with liver necrosis and inflammation19 (Extended Data Fig. 2a, b, arrows). There was a prominent ductular reaction in β1-integrinfl/fl livers compared with control β1-integrinWT livers, with increased biliary cytokeratin-19-positive (CK19pos) cells and alpha smooth muscle actin-positive (αSMApos) myofibroblasts. CK19pos cells were distributed throughout the parenchyma (Extended Data Fig. 2c-e). β1-Integrinfl/fl livers had small areas of tdTom-negative (tdTomneg) hepatocytes adjacent to the portal tracts (Extended Data Fig. 2c, f). Furthermore, β1-integrinfl/fl livers contained more Ki67-positive (Ki67pos) hepatocytes (proliferating marker) and more p21-positive (p21pos) hepatocytes (cell-cycle arrest marker) (Extended Data Fig. 2g, h).

We damaged β1-integrinWT and β1-integrinfl/fl livers with three independent regimes: DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) diet, a model of cholestatic liver disease; methionine- and choline-deficient (MCD) diet, a model of non-alcoholic steatohepatitis and thioacetamide (TAA); and a model of liver fibrosis20. We then studied the regenerative dynamics (Fig. 1a). After DDC injury, all mice lost weight but β1-integrinfl/fl mice had delayed weight recovery. β1-Integrinfl/fl mice had prolonged hepatocellular Ki67 and p21 expression and elevated serum markers of liver injury (Extended Data Fig. 3a–d), indicating that, after β1-integrin loss, hepatocytes are unable to regenerate the injured liver efficiently. Hepatocyte Ki67 and p21 expression was rarely co-localized (Extended Data Fig. 3e). β1-Integrinfl/fl livers had increased liver necrosis and recovered slowly from DDC injury (Extended Data Fig. 3f, g, asterisks). There were increased ductular reactions after hepatocyte β1-integrin loss, demonstrating that the DDC-induced injury remains unresolved in the absence of hepatocyte β1-integrin (Fig. 1b). Persistent ductular reactions were also found in β1-integrinfl/fl livers that were injured with MCD and TAA (Fig. 1c). After hepatocyte β1-integrin loss, DDC-induced ductular reactions had atypical biliary cells, not confined to luminal structures (Fig. 1e, arrowheads, and Extended Data Fig. 4a, arrows). Ductular reactions in β1-integrinfl/fl livers were surrounded by αSMApos myofibroblasts that peaked in number by day 7 recovery; however, the degree of fibrosis increased throughout recovery (Fig. 1d, e and Extended Data Fig. 3h, i).

To determine whether non-hepatocytes regenerated hepatocytes, we examined tdTom expression. After liver injury and recovery with DDC, MCD, and TAA, 20–30% of hepatocytes positive for CYP2D and HNF4α (mature hepatocyte markers) were tdTompos (Fig. 1f–h and Extended Data Fig. 4a, b). Small, proliferative tdTompos hepatocytes were identified from day 7 and increased significantly until day 14 (Extended Data Fig. 4c-e). tdTompos hepatocytes were normally found adjacent to SOX9pos and CK19pos ductal cells, and the number of tdTompos hepatocytes decreased with distance from ductular reactions (Fig. 2a, b). SOX9pos/tdTompos/HNF4apos cells could be seen at the border between unlabelled parenchyma and ductular reactions (Fig. 2a), putatively representing an intermediate cell state between...
Hepatocyte β1-integrin ablation combined with liver injury amplifies ductular reaction, and hepatocytes regenerate from a non-hepatocyte source. a, Experimental strategy to stimulate liver regeneration with hepatotoxic agents. b–d, Quantification of CK19pos cells or αSMApos cells before, during, and after injury (n = 5 DDC, n = 3 MCD, and n = 2 TAA; n = 3 for pre-injury αSMA analysis). PFV, per field of view. e, Confocal CK19/αSMA immunofluorescence; invasive biliary cells (arrowheads), ducts with lumens (arrows). f, h, Quantification of tdTompos/HNF4αpos hepatocytes before, during, and after injury (n = 5 DDC, n = 3 MCD, and n = 2 TAA). g, Confocal tdTom immunofluorescence of CYP2D and HNF4α hepatocytes (n, number of mice per condition; experiments were performed once or twice). Scale bars, 100 μm. Data are mean ± s.e.m.; ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001; two-way analysis of variance (ANOVA), Bonferroni post-tests.

In humans, increased expression of p21 in hepatocytes is seen in chronic liver disease, suggesting that hepatocyte replicative potential becomes exhausted1,2. We sought to reproduce this in our mouse models of liver injury and regeneration by impairing hepatocyte proliferation to mobilize a traceable, biliary-derived hepatocyte regenerative response. To achieve this, we used a p21 coding region inserted into an AAV8 vector downstream of a thyroxine-binding globulin (TBG) promoter22 to overexpress p21 in hepatocytes (Fig. 3a). Liver Cdkn1a messenger RNA (mRNA) expression was six times higher in the AAV8-p21 injected group (Fig. 3b). Hepatocyte p21 expression was observed after injection of the AAV8-p21 DNA (Fig. 3c). Liver histology remained normal (Extended Data Fig. 6a, b). Proliferating non-parenchymal cells were seen close to the portal tracts (Fig. 3d). Additionally, no significant changes in ductular reaction or collagen deposition were detected after p21 overexpression (Extended Data Fig. 6c, d). To confirm that p21 overexpression was sufficient to inhibit hepatocyte proliferation, we induced acute liver injury after AAV8 injection (Fig. 3e). Ki67pos/HNF4αpos hepatocytes were only present in mice that received the AAV8-ctrl vector (Fig. 3f), and p21 expression was increased in the AAV8-p21 group (Fig. 3f, g). No significant changes in necrotic area were observed (Fig. 3h), although serum transaminases were higher in the AAV8-p21 group (Fig. 3i).
We used the CK19CreERTtdTomato<sup>LSL</sup> lineage tracing model to test whether cholangiocytes gave rise to hepatocytes in various diet-induced liver injuries combined with p21 overexpression (Fig. 4a). After the CDE, MCD, or DDC injury regimes (Fig. 4a and Extended Data Fig. 8a, f), tdTom-expressing hepatocytes were observed in the AAV8-p21 group (Fig. 4b and Extended Data Fig. 8b, c, g, h). tdTom<sup>pos</sup> cells contributed to 6.12 ± 0.3% of the liver 2 weeks after injury (Fig. 4b). Considering that the KrtrlCreERTtdTomato<sup>LSL</sup> system labels approximately 40% of all biliary cells, we corrected for this limitation in recombination efficiency, predicting that biliary cells differentiate into approximately 15.3% of hepatocytes in this model. Periodic acid–Schiff staining of serial liver sections showed the tdTom<sup>pos</sup> hepatocytes stored glycogen (Fig. 4c). Other hepatocyte markers such as HNF4α, glutamine synthetase, and CYP2D were expressed by these cholangiocyte-derived tdTom<sup>pos</sup> hepatocytes (Fig. 4d and Extended Data Figs 8d, i, and 9a). These cholangiocyte-derived hepatocytes were present 10 weeks after injury (Extended Data Fig. 9b–e). Perportal tdTom<sup>pos</sup>, SOX9<sup>pos</sup> hepatocytes were adjacent to the tdTom<sup>pos</sup>, SOX9<sup>pos</sup> ductular reactions, suggesting differentiation of SOX9<sup>pos</sup> ductular cells into hepatocytes (Fig. 4e and Extended Data Fig. 8e). tdTom<sup>pos</sup> hepatocytes expressing HNF4α and zone 1 marker E-cadherin were directly adjacent to the tdTom<sup>pos</sup> and PanCK<sup>pos</sup> cholangiocytes, in keeping with their ductular origin (Fig. 4f). To test the proliferative capacity of the cholangiocyte-derived tdTom<sup>pos</sup> hepatocytes, we performed 50% partial hepatectomy after MCD injury/recovery (Extended Data Fig. 9f). Fewer hepatocytes in AAV8-p21 group were proliferative after hepatectomy (Extended Data Fig. 9g), and perportal tdTom<sup>pos</sup> Ki67<sup>pos</sup> E-cadherin<sup>pos</sup> proliferating hepatocytes were observed in the AAV8-p21 group (white arrows) (Extended Data Fig. 9h). These results confirm the direct contribution of labelled cholangiocytes to hepatocyte regeneration during injury when hepatocyte proliferation is inhibited. To compare hepatocytes of different origins, we isolated non-hepatocyte-derived hepatocytes from the β1-integrin<sup>fl/fl</sup> model (tdTom<sup>neg</sup>) and cholangiocyte-derived hepatocytes from the AAV8-p21 model (tdTom<sup>pos</sup>) (Extended Data Fig. 10a–c), and compared them against hepatocytes and cholangiocytes from injured WT mice. Gene expression between these cells was assessed with a drug metabolism quantitative PCR (qPCR) array and RNA sequencing (RNA-seq). Global transcriptional states of the biliary-derived hepatocytes from AAV8-p21-treated livers and tdTom<sup>neg</sup> hepatocytes from the β1-integrin<sup>fl/fl</sup> model were highly similar to hepatocytes and distinct from ductal cells (Fig. 4g and Extended Data Fig. 10d–f). tdTom<sup>pos</sup> hepatocytes clustered closely with WT hepatocytes (Fig. 4h). Differences in the newly formed tdTom<sup>neg</sup> hepatocytes from the β1-integrin<sup>fl/fl</sup> model may be explained by the continued presence of defective β1-integrin<sup>fl/fl</sup> hepatocytes in the liver and their effect upon the tissue microenvironment (Extended Data Fig. 10e, f).

In summary, we have studied impaired hepatocyte regeneration in two independent systems: hepatocyte 31-integrin ablation and p21 overexpression. Both models triggered ductular reactions followed by clusters of cholangiocyte-derived hepatocytes. Multiple hepatocyte sub-populations have been suggested as regenerative under homeostasis<sup>3,24</sup> or liver injury<sup>34</sup>. However, our findings show that while ductular reactions can be generated after injury, impaired hepatocyte regeneration is required for cholangiocytes to form hepatocytes. The mechanism of effect of the two models is different: 31-integrin ablation causes hepatocyte necrosis, p21 expression, and inhibition of growth factor signalling<sup>35</sup>, viral p21 overexpression directly inhibits hepatocyte proliferation. The common factor between the two models is impaired hepatocyte regeneration. This explains previous conflicting data from mouse, rat, and human disease, where the lack of differentiation seen in lineage-tracing models was probably due to ongoing effective hepatocyte-mediated regeneration. The full potential of cholangiocyte-derived hepatocyte regeneration may be higher as these are short-term injury models compared with human liver disease. These systems have now

To induce ductular reactions on the background of impaired hepatocyte proliferation, we used the choline-deficient ethionine-supplemented (CDE) diet combined with AAV8-p21 or AAV8-ctrl (Extended Data Fig. 7a). Hepatocyte proliferation was inhibited in mice in the AAV8-p21 group (Extended Data Fig. 7b, d). Proliferating cells observed in the AAV8-p21 injected group were HNF4α<sup>neg</sup> (Extended Data Fig. 7c). After CDE injury, there was a trend to increased ductular reactions in the AAV8-p21 group (Extended Data Fig. 7e). The AAV8-p21 group had persistent hepatocellular p21 expression 2 weeks after injury compared with control (Extended Data Fig. 7f). mRNA analysis showed a 5-fold increase in p21 expression, and liver-damage-related genes such as Igfbp1 and Tgfb1 increased 20-fold and 2-fold respectively. In addition, a 4-fold increase in the ductular reaction gene Krtrl was found (Extended Data Fig. 7g). Together, these findings demonstrate that AAV8-p21 administration causes hepatocyte p21 accumulation and impaired hepatocyte proliferation after injury.
**Figure 3** | Inhibition of hepatocyte proliferation after AAV8-p21 injection. a, Schematic representing the AAV8-p21 construct. b, Cdk1a whole-liver RNA expression after AAV8 injection (n = 3 mice). c, Hepatocyte p21 immunohistochemistry of AAV8-injected mice. d, Ki67 immunostaining of AAV8-injected livers. e, Experimental design of the AAV8-p21 with CCl4 regime. f, Top: Ki67/HNF4α immunofluorescence after AAV8 and CCl4 injection, upper panel. Bottom: p21 immunohistochemistry after CCl4 injection. g, Quantification of Ki67pos hepatocytes in mice injected with CCl4 after AAV8 injection (n = 3 mice). h, Necrotic area 48 h after CCl4 injection (n = 7 mice). i, Mouse serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) after CCl4. Scale bars, 100 μm; n = 3 mice. Data are representative of one or two independent experiments. Experiments were performed twice. Data are mean ± s.e.m. Mann–Whitney U-test, *P < 0.05.

**Figure 4** | Emergence of ductular-derived hepatocytes after chronic liver injury when hepatocyte proliferation is impaired. a, Schematic of the MCD/recovery injury diet regime. b, tdTom immunohistochemistry on Krt19-tdTomato-livers after MCD recovery regime. High-magnification images and quantification of tdTom-positive cells (insets) (n = 4 mice). c, Immunostaining of tdTom and periodic acid–Schiff staining on serial liver sections of AAV8-injected mice. d, Immunofluorescent double staining of HNF4α (green, top), glutamine synthetase (GS; green, middle), CYP2D (green, bottom), and tdTom (red). e, Double immunohistochemistry of tdTom (red), SOX9 (green, top), PanCK (green, bottom). f, Confocal images of serial liver sections of AAV8–injected mice; tdTom (red), HNF4α (green, left), PanCK (green, right), E-cadherin (E-cad; grey) (n = 4 mice). g, h, Pearson’s correlation plot with hierarchical clustering and three-dimensional principal component analysis for total transcriptional landscapes (n = 3 mice). Scale bars, 100 μm. Data are representative of one or two independent experiments; experiments were repeated at least twice, except g and h were only performed once. Data are mean ± s.e.m. Mann–Whitney U-test, *P < 0.05.
identified the cellular sources of regeneration in severely injured adult liver. This will facilitate studies into the signals controlling this regenerative pathway, aiding the development of liver regeneration therapies.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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METHODS

Animal models. The animals in this study were on a C57BL/6J background. Both male and female mice were used. Animals were housed in a specific pathogen-free environment and kept under standard conditions with a 12 h day/night cycle and access to food and water ad libitum. All animal experiments were performed under procedural guidelines, severity protocols, and with ethical permission from the University of Edinburgh Animal Welfare and Ethical Review Body and the UK Home Office.

To delete 31-integrin in vivo, we used the 31-integrinfloxed mice (The Jackson Laboratory); in this model exon 3 of 31-integrin gene is flanked by loxP sites17. To lineage trace 31-integrinfloxed cells, we crossed this strain with the R26RtdTomatoSL mouse (The Jackson Laboratory).

Genotyping was performed by the Transnetyx genotyping service.

Mice between 7 and 10 weeks of age had Cre-mediated recombination induced with AAV8.TBG.P.I.Cre.RtBG (Penna vector core, CS0644). Control, null experiments used AAV8.TBG.P.I.null.RtBG (Penna vector core, CS0253). Viruses were administered by tail vein injection at a concentration of 2.5 × 1011 genome copies per millilitre. AAV8 viruses were diluted in sterile PBS. Mice were given a 2-week washout period before being placed on toxic injury diets.

Small interfering RNA (siRNA) formulated into lipidoid nanoparticles was used to silence Igf31 mRNA in vivo. These siRNA nanoparticles have previously been shown to specifically suppress hepatocyte 31-integrin expression16,21. Doses (0.5 mg per kg body weight) were administered intravenously via the tail vein to each mouse every 5 days according to the schematic in Fig. 2c.

The Kr19T2DTomato22 mice were induced by three individual intraperitoneal injections of tamoxifen (20 mg ml−1, Sigma UK) at a dose of 4 mg. K19Cre mice received 2 weeks of normal diet after the last tamoxifen injection before starting an injury diet regimen.

To induce liver injury, mice were given 0.1% DDC mixed with Rat and Mouse chow (Special Diet Services) and overnight at 4 °C using the antibodies listed in Supplementary Table 1. Primary antibodies were detected using fluorescent-labeled secondary antibodies (Alexa Fluor 488/Alexa Fluor 555 and Alexa Fluor 647) and were quantified using haematoxylin and eosin histochemistry and the trainable WEKA segmentation tool in Fiji Image.

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Histology, immunohistochemistry, and immunofluorescence. Formalin-fixed tissue embedded in paraffin. Sections (4 μm) were blocked with protein block (Speckle Glue, Vector Laboratories) and stained overnight at 4 °C using the antibodies listed in Supplementary Table 1. Primary antibodies were detected using fluorescent-conjugated secondary antibodies (Alexa Fluor 488/Alexa Fluor 555 and Alexa Fluor 657; Invitrogen). Sections were stained with DAPI (4’,6-diamidino-2-phenylindole) and mounted with Fluoromount-G (SouthernBiotech). For DAB-stained sections, tissue was blocked with Bloxall (Vector), Avidin/Biotin block (Invitrogen), and protein block. Primary antibodies were incubated overnight at 4 °C. Species-specific secondary biotinylated antibodies (Vector, R.T.U. Vectastain, ABC reagent (Vector), and DAB chromogen (Dako)) were used to detect the primary antibody; Haematoxylin counterstain was used alongside the DAB.

When two antibodies from the same species were used, Soy9/RFP/HNA4a immunofluorescent stain primary antibodies were applied sequentially. The first antibody to be applied, anti-RFP, was detected using a species-specific secondary antibody (Dako) conjugated to HRP and a Perkin Elmer TSA Plus Cyanine 3 signalling amplification kit (NEL748B001KT). This was followed by a second antigen retrieval to denature any antibodies in the tissue and prevent cross-reaction with the following antibody application.

Haematoxylin and eosin stains were automatically produced using a Shandon TRizol Automated Slide Stainer. PicroSirius Red stains used reagents from Sigma Aldrich: picric acid, catalogue number P6744-1GA; fast green, catalogue number F7258-25G; direct red, catalogue number 36558-25G. Staining was done according to the manufacturer’s instructions.

Microscopy and cell counting. For confocal microscopy, either a Leica SPE inverted microscope or a Zeiss LSM 780 confocal microscope were used and images were deconvoluted using Fiji ImageJ. Brightfield images were taken using a Nikon Eclipse e600 microscope and Retiga 2000R camera (Q-Imaging) and acquired with Image-Pro Premier software. PicroSirius Red analysis used an Axiocan S Z.1 (Zeiss) to acquire tiled images at > 20 magnification. De-tiled images were then analysed using a standard colour threshold in Fiji Image J. Necrotic areas were quantified using haematoxylin and eosin histochemistry and the trainable WEKA segmentation tool in Fiji Image.

Fluorescent tiled images were generated on a Perkin Elmer Operetta high-content imaging system; subsequent image staining was done on Fiji Image using the pairwise stitching plugin22. DAPI, Alexa Fluor 488, and Alexa Fluor 555 were detected using excitation band-pass filters of 360–400, 460–490, 520–550 nm and emission band-pass filters of 410–480, 500–550, 560–630 nm, respectively.

In cases where manual quantification was performed (including animal experiments), counts were performed on blinded slides and more than 20 consecutive non-overlapping fields at ×100 or ×200 magnification

Single-cell liver tissue analysis. Images were acquired in up to four fluorescent channels at ×10 magnification on a Perkin Elmer Operetta high-content imaging system and subsequently analysed using the Columbus software. An average of 25 images were taken per liver section. One field of view corresponded to 1.37 mm2. DAPI-stained nuclei were identified on the basis of pixel intensity using method ‘M’ with approximately 7,000 nuclei identified per field. Nuclear size and morphology were then determined. Illumination correction and background normalization were performed using the sliding parabola module. Depending on the experiment, nuclei were then assigned as positive or negative on the basis of the mean pixel intensity in the corresponding channel in either the nucleus (HNF4α, tdTom, KI67, P21, and PCNA) or a 7 μm thick region surrounding the nucleus (CK19 and αSMA). For each experiment, identical thresholds were used in all images for assigning nuclei to a specific population.

Protein homogenates and western blots. Protein was isolated from whole liver. Small, 1–2 mm3 pieces of liver were homogenized using a Tissue-Tearor (Biospec Products) and lysis buffer (150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2× Protease Inhibitor Cocktail (Sigma Aldrich)). Samples were mixed at 4 °C for 30 min and then centrifuged for 10 min at 20,000g. The aqueous supernatant was removed and the protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Proteins were separated using SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes with anti-β-actin (Millipore, MAB1997) 1/1,000 and anti-β-actin (Cambridge Biosciences) 1/2,000 were used to detect proteins.

Isolation of bilary ducts and hepatocytes, and FACS. Hepatocytes and biliary ducts were isolated using the classical perfusion protocol. Briefly, animals received an overdose of anesthesia followed by laparotomy: the portal vein was cannulated and injected with Liver Perfusion Medium (Gibco) and Liver Digest Medium (Gibco). For duct isolation, ducts were digested and isolated as previously described8. For hepatocyte isolation, the liver was removed and mechanically disaggregated. The resulting dissociated cells were filtered through a 70 μm filter (BD Biosciences). Hepatocytes were purified with a density gradient centrifugation8, which isolated cells with hepatocyte morphology and expression of CYP2D6 (a mature hepatocyte marker) at a purity greater than 99% (ref. 7). Briefly, cells were centrifuged through Percoll (Sigma) in PBS. Cells were spun at 750g for 20 min. The hepatocyte layer, between the 1.08 and 1.12 mg ml−1 Percoll layers, was collected for FACS. Purified hepatocytes were sorted on a BD Biosciences Flow Cytometer. Sorted cells where lysed and the RNA was extracted.

RNA isolation and qPCR with reverse transcription. Liver tissue was homogenized in TRizol (Life Technologies). Homogenates were mixed with chloroform (1:5 ratio chloroform:TRizol) and centrifuged at 4 °C, 1,200g, for 15 min. The aqueous supernatant was removed and mixed 1:1 with 70% ethanol. RNA was extracted using a Qiagen RNeasy Mini Kit according to the manufacturer’s instructions. Reverse transcription and qPCR were performed using Qiagen QuantiTect and QuantiFast reagents on a LightCycler 480 II (Roche). Commercial primers and probes were purchased from Qiagen’s QuantiTect range were used: Cdkn1a (Qiagen QT00137053), Igfbp1 (Qiagen QT001114716), Tgfb1 (Qiagen QT00145250), krt19 (Qiagen QT00156667), and peptidylprolyl isomerase A

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Gene expression was normalized to the housekeeping gene, PPIA. Samples were run in triplicate. The RT² Profiler PCR Array, Drug Metabolism: Phase I Enzymes was purchased from Qiagen (330231 PAMM-068Z), and complementary DNA (cDNA) was synthesized from extracted RNA using the RT² First Stand Kit (Qiagen; 330401) according to the manufacturer’s instructions. The RT² Profiler PCR Array was run on a Roche LightCycler 480 II with RT² SYBR Green qPCR Mastermix (Qiagen; 330500), according to the manufacturer’s instructions. Analysis were performed according to the manufacturer’s templates and guidelines.

Whole-genome RNA-seq. Samples were treated with DNase (Ambion), and sample integrity verified on an Agilent Bioanalyser with the RNA Nano chip. Illumina Tru-seq paired-end strand-specific sequencing (Illumina, USA) was performed on a NextSeq-550 sequencer (Edinburgh Clinical Research Facility, Western General Hospital, Edinburgh, UK). Total RNA (500 ng) underwent ribosomal RNA depletion before purification, fragmentation, random hexamer cDNA generation, and purification with AMPure XP beads (Beckman-Coulter, USA). Multiple indexing adapters were ligated to double-stranded cDNA with subsequent hybridization onto flow cells, and DNA fragment enrichment by 13-cycle PCR for sequencing. Completed libraries were quantified by qPCR using a KAPA Illumina Library Quantification Kit (Illumina, USA) before multiplexing in two equimolar pools and running on two flow cells on an Illumina NextSeq 550. The resulting FastQ files were mapped to the reference genome (mm9) using the Tophat alignment tool (version 2) on Illumina Basespace software and reads per kilobase per million (RPKM) scores calculated for each gene. Differential gene expression was done using DEseq with cutoffs of log2(fold change) > 2 and adjusted P < 0.05 within replicates applied. Global analysis of total RPKM data sets to assess overall transcriptional states was done by calculating and plotting Pearson’s correlation scores visualized as a heatmap with Euclidian and Ward clustering applied. Principal component analysis plots were also performed with the use of Illumina Basespace software. Plots for Pearson’s correlation scores with hierarchical clustering were also performed on all genes displaying significant gene expression changes relative to the control set. Visual examples of the transcriptional data were available from the corresponding author upon reasonable request.

Reproducibility of experiments. Routinely, qPCR experiments were performed in technical triplicates of multiple biological replicates. For representative images, three or four liver lobes were examined histologically in at least three biological replicates. For representative images, three or four liver lobes were examined histologically in at least three biological replicates; further details referring to the specific numbers of biological replicates for each experiment can be found in the figure legends.

Data availability. Data generated during this study are available in the Gene Expression Omnibus under accession number GSE98034. All other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | AAV8.TBG.Cre labelled and deleted β1-integrin in 99.5% of hepatocytes. a, tdTom, biliary-specific cytokeratin 19 (CK19), and hepatocyte-specific HNF4α immunofluorescence in AAV8.TBG.Cre/null-treated livers 2 weeks after AAV administration. Cre-treated HNF4αpos hepatocytes are tdTompos in contrast to the null-treated; CK19pos ductal cells are located at the portal tract (PT). Confocal images. b, Liver tissue analysis quantifying tdTompos, HNF4αpos hepatocytes 2 weeks after AAV8.TBG.Cre/null administration. AAV8.TBG.Cre, n = 7; AAV8.TBG.null, n = 3. c, Hepatocellular β1-integrin immunofluorescence greyscale image and dual image with membranous β-catenin, 2 weeks after AAV8.TBG.Cre administration; arrows identify β1-integrin in the hepatocyte cell membrane, arrowheads identify non-parenchymal β1-integrin staining. Confocal images. d, Two weeks after AAV8.TBG.Cre administration; whole-liver β1-integrin. Western blot. e, Two weeks after AAV8.TBG.Cre administration; whole-liver QPCR for β1-integrin (n = 3; n, number of mice per group, experiment was repeated twice). Scale bars, 100μm. Data are mean ± s.e.m.; two-tailed unpaired t-test; *P = 0.05.
Extended Data Figure 2 | Ablation of β1-integrin caused liver damage, ductular reaction, and small patches of unlabelled periportal hepatocytes. a, Serum enzyme-linked immunosorbent assay for markers associated with liver function 9 weeks after AAV8.TBG.Cre administration (n = 3). b, Nine weeks after AAV8.TBG.Cre administration; anti-HMGB1 DAB immunohistochemistry. Arrowheads identify nuclear HMGB1, arrows highlight cytoplasmic HMGB1. c, Nine weeks after AAV8.TBG.Cre; αSMA/CK19, tdTom/HNF4α, and glutamine synthetase/tdTom immunofluorescence; arrows point to areas with ductular reaction. d, Liver tissue analysis quantifying CK19pos cells 9 weeks after AAV8.TBG.Cre administration (n = 3). e, Liver tissue analysis quantifying αSMApos cells 9 weeks after AAV8.TBG.Cre administration (n = 3). f, Liver tissue analysis quantifying tdTompos, HNF4αpos hepatocytes in livers 9 weeks after AAV8.TBG.Cre administration (n = 3). g, Liver tissue analysis quantifying Ki67pos, HNF4αpos hepatocytes 9 weeks after AAV8.TBG.Cre administration (n = 3). h, Liver tissue analysis quantifying p21pos, HNF4αpos hepatocytes 9 weeks after AAV8.TBG.Cre administration (n = 3; n, number of mice per condition, the experiment was performed once). Scale bars, 100 μm. Data are mean ± s.e.m.; two-tailed unpaired t-test; *P = 0.05, **P = 0.01.
Extended Data Figure 3  |  Hepatocyte β1-integrin deletion delayed and altered the regenerative response after treatment with the liver injury diet DDC. a, DDC diet and recovery; serum enzyme-linked immunosorbent assay for markers associated with liver function (n = 5). b, Body weight measurements during DDC diet and the subsequent recovery (n = 5). c, Liver tissue analysis quantifying prolonged Ki67pos/HNF4αpos hepatocytes after DDC injury (n = 5). d, Liver tissue analysis quantifying p21pos/HNF4αpos hepatocytes after DDC injury (n = 5), and representative p21/HNF4α immunofluorescence in samples 7 days after DDC injury. e, p21/Ki67 immunofluorescence 7 days after DDC injury. f, Representative haematoxylin and eosin histochemical stain of sections after DDC injury; asterisks highlight necrotic areas, arrows highlight small cellular infiltrate. g, Morphometric quantification of necrotic area from haematoxylin and eosin sections in DDC-treated samples at peak injury and recovery. h, Fibrosis analysis; percentage of pixels PicroSirius Red (PSR)-positive before, during, and after DDC injury (n = 5, except pre-injury PicroSirius Red, which is n = 3). i, Representative images of PicroSirius Red histochemistry on samples from day 42 of recovery after DDC diet (n, number of mice per condition, the experiment was performed once or twice). Scale bars, 100 μm. Data are mean ± s.e.m.; two-tailed unpaired t-test; two-way ANOVA, Bonferroni post-tests. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Figure 4 | β1-Integrin ablation and DDC-induced injury resulted in large areas of tdTom<sup>neg</sup> hepatocytes. a, tdTom/CK19 immunofluorescence; tiled images of β1-integrin<sup>fl/fl</sup> and β1-integrin<sup>WT</sup> livers after DDC diet and 14 days of recovery. 1–4, Enlarged regions of interest: 1, invasive CK19-positive cells (arrows); 2, tdTom<sup>neg</sup> regenerative nodule; 3, 4, fully recovered β1-integrin<sup>WT</sup> liver with typical biliary ducts (arrowheads). b, Separate channels from a merged CYP2D and tdTom immunofluorescent confocal image in Fig. 1g, showing CYP2D expression between tdTom<sup>pos</sup> and tdTom<sup>neg</sup> hepatocytes. c–e, Liver tissue analysis and a representative PCNA/HNF4α/tdTom immunofluorescent image in β1-integrin<sup>fl/fl</sup> mice at day 14 recovery after DDC injury; quantifying PCNA expression and nucleus size in both tdTom<sup>pos</sup> and tdTom<sup>neg</sup> hepatocytes (HNF4α<sup>pos</sup>) (n = 8 mice analysed, the experiment was performed once). f, Liver tissue analysis in β1-integrin<sup>fl/fl</sup> mice at day 14 recovery after DDC injury; quantifying tdTom<sup>pos</sup> HNF4α<sup>pos</sup> hepatocytes adjacent to the glutamine-synthetase-positive central veins (GSP<sup>pos</sup> CV) (n = 7 mice analysed, the experiment was performed once). g, tdTom/glutamine synthetase/CK19 immunofluorescence in mice at day 14 recovery after DDC diet; β1-integrin<sup>fl/fl</sup> liver has patches of tdTom<sup>neg</sup> hepatocytes connected to CK19<sup>pos</sup> biliary epithelium, tdTom<sup>pos</sup> hepatocytes remain arranged around the glutamine-synthetase-positive central veins. Data are mean ± s.e.m.; **P < 0.01, paired t-test.
Extended Data Figure 5 | \(K_{19}^{CoeRT}tdTomato^{LSL}\) strictly labelled 40% of the biliary epithelium. Three 4 mg doses of tamoxifen followed by a 14-day wash out period. a, Representative images of CK19/tdTom dual immunofluorescence. b, Quantification of CK19\textsuperscript{pos} biliary epithelial cells that were tdTom-positive (\(n = 8\) mice analysed, the experiment was performed once). Scale bars, 100 \(\mu\)m. Data are mean ± s.e.m.
Extended Data Figure 6 | Analysis of AAV8-p21-injected livers.

a, Experimental design of the AAV8-p21 injection. b, Haematoxylin and eosin staining on the livers of AAV8-injected mice. c, Quantification and representative images of immunostaining for PanCK<sup>pos</sup> cells of the AAV8 vector-injected liver. d, PicroSirius Red analysis of the AAV8 vector-injected livers. Scale bars, 100 μm; n = 5 mice. Experiments were performed twice. Data are mean ± s.e.m. Mann–Whitney U-test, *P < 0.05.
Extended Data Figure 7 | Analysis of AAV8-p21-injected livers after CDE injury regime. 

a, Schematic representation of the CDE recovery regime. 
b, Immunostaining of Ki67 (red) and HNF4α (green) on AAV8-injected mice during CDE injury. White arrows denote Ki67- and HNF4α-double-positive hepatocytes. 
c, Split channel images of DAPI, Ki67, and HNF4α on liver sections of AAV8-p21-injected mice after CDE injury. 
d, Ki67 (red) and HNF4α (green) immunohistochemistry of the liver of mice injected with AAV8-p21 and control 2 weeks after CDE injury. 
e, PanCK immunohistochemistry and quantification of PanCK* cells of the liver of AAV8-injected mice during and 2 weeks after CDE diet-induced injury. 
f, The p21 immunohistochemistry on AAV8-injected mice during and after CDE injury. Insets show high-magnification images. 
g, Whole-liver Cdkn1a, Igfbp1, Tgfβ1, and Krt19 mRNA expression of AAV8 vector-injected mice 2 weeks after CDE injury. Scale bars, 100 μm; n = 4 mice. Experiments were performed once. Data are mean ± s.e.m. Mann–Whitney U-test, *P < 0.05.
Extended Data Figure 8 | Analysis of the Krt19Cre;tdTomatoLSL mice which received AAV8-p21 injection followed by DDC or CDE recovery regime. a, Experimental design of the AAV8-p21 injection followed by DDC recovery regime. b, c, tdTom staining of Krt19Cre;tdTomatoLSL mice that received AAV8-p21 injection followed by DDC recovery regime. d, HNF4α (green) and tdTom (red) staining on the Krt19Cre;tdTomatoLSL mice that received AAV8 injections and were treated with the DDC recovery regime. e, SOX9 (green) and tdTom (red) staining on the Krt19Cre;tdTomatoLSL mice that received AAV8 injections and were treated with the DDC recovery regime. f, Schematic representation of the experimental design of the AAV8-p21 injection followed by CDE recovery regime. g, Epifluorescence images of the liver of the mice that received AAV8-ctrl or AAV8-p21 injections followed by the CDE recovery regime. h, Immunostaining for tdTom of mice that received AAV8-ctrl or AAV8-p21 injections followed by the CDE recovery regime. i, tdTom (red) and HNF4α (green) immunofluorescent double staining of mice that received AAV8-ctrl or AAV8-p21 injections followed by the CDE recovery regime. N = 4 mice, experiments were performed once. Scale bars, 100 μm.
Extended Data Figure 9 | Analysis of the Krt19<sup>Cre</sup> tdTomato<sup>LSL</sup> mice that received AAV8-p21 injection followed by MCD recovery regime. 

**a**, Representative immunofluorescent images showing split channels of tdTom, HNF4α, and DAPI of mice that received AAV8-ctrl or AAV8-p21 injections followed by the MCD recovery regime. 

**b**, Schematic representation showing the experimental design of the AAV8-p21 injection followed by a MCD long recovery regime. 

**c, d**, tdTom staining of Krt19<sup>Cre</sup> tdTomato<sup>LSL</sup> mice that received AAV8-p21 injection followed by MCD long recovery regime. 

**e**, tdTom (red) and HNF4α (green) immunofluorescent double staining and quantification of tdTom<sup>pos</sup> HNF4α<sup>pos</sup> hepatocytes in that mice that received AAV8-ctrl or AAV8-p21 injections followed by the MCD long recovery regime. 

**f**, Representation of partial hepatectomy performed after the MCD recovery regime. 

**g, h**, Quantification and immunofluorescent double staining of HNF4α (red) and Ki67(green) of mice that received 50% partial hepatectomy. 

**h**, Confocal microscopy images of mice that received 50% partial hepatectomy; tdTom (red), Ki67 (green), E-cadherin (grey). 

N = 4 mice, experiments were performed twice. Scale bars, 100 μm. Data are mean ± s.e.m.
Extended Data Figure 10 | Comparison between ductular-derived hepatocytes and hepatocytes regenerated through self-duplication.

a, Experimental design to isolate WT hepatocytes/ducts and ductular-derived hepatocytes from a regenerated liver. B, FACS gating strategy to isolate tdTom^pp^ and tdTom^neg^ hepatocytes. c, Cytospins and tdTom/HNF4α immunofluorescence of FACS-sorted cells. d, Visual examples of average RNA-seq transcriptional reads in sample groups across select loci. Scales referring to normalized read counts are displayed on the right of each plot. RNA-seq annotated genes are plotted in black below. e, RNA-seq generated Z-score heatmaps with hierarchical clustering across specific gene sets. Heatmaps display gene expression levels normalized to each gene; yellow, higher expression; blue, lower expression; n = 3. f, qPCR array for phase 1 drug metabolism enzymes (n = 3 mice). Experiments were performed once. Scale bars, 100μm.
Corrigendum: Cholangiocytes act as facultative liver stem cells during impaired hepatocyte regeneration

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In Extended Data Fig. 10b of this Letter, the axes of the single-cell gating (middle panel) FACS plots were mislabelled. Single cells were gated using forward scatter area (FSC-A) against height (FSC-H) on a linear scale, instead of side scatter area (SSC-A) against height (SSC-H) on a log scale. This does not affect the conclusion drawn. This figure has been corrected in the online versions of the Letter, and the original incorrect figure is provided as Supplementary Information to this Corrigendum, for transparency.

In addition, a reference was inadvertently omitted to earlier work in zebrafish, which should have appeared associated with the sentence ‘Biliary cells in zebrafish models have been shown to regenerate the liver after massive hepatocytes loss’. This has been added as ref. 25 in the Letter, and citations in the Methods section (refs 26–28) have been renumbered. The original Letter has been corrected online.

Supplementary Information is available in the online version of this Corrigendum.

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