Hepatic progenitor cells of biliary origin with liver repopulation capacity

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Hepatocytes and cholangiocytes self-renew following liver injury. Following severe injury hepatocytes are increasingly senescent, but whether hepatic progenitor cells (HPCs) then contribute to liver regeneration is unclear. Here, we describe a mouse model where the E3 ubiquitin ligase Mdm2 is inducibly deleted in more than 98% of hepatocytes, causing apoptosis, necrosis and senescence with nearly all hepatocytes expressing p21. This results in florid HPC activation, which is necessary for survival, followed by complete, functional liver reconstitution. HPCs isolated from genetically normal mice, using cell surface markers, were highly expandable and phenotypically stable in vitro. These HPCs were transplanted into adult mouse livers where hepatocyte Mdm2 was repeatedly deleted, creating a non-competitive repopulation assay. Transplanted HPCs contributed significantly to restoration of liver parenchyma, regenerating hepatocytes and biliary epithelia, highlighting their in vivo lineage potency. HPCs are therefore a potential future alternative to hepatocyte or liver transplantation for liver disease.

Liver transplantation is the only effective cure for advanced liver disease. However, a global shortage in donor organs means that many patients die waiting for suitable organs. Hepatocyte cell therapy is an attractive alternative to liver transplantation1-2. Hepatocytes have a high replicative potential in vitro3,4, however, hepatocyte isolation is technically demanding and requires cadaveric liver donation, and hepatocytes cannot be expanded effectively in vitro5.

Ductular reactions are activated in chronic liver injury and are thought to include putative hepatic progenitor cells (HPCs) with the potential to regenerate hepatocytes and cholangiocytes6-7. However, the significance of this regenerative pathway is unclear. Recent studies have sought to identify HPCs using surface markers8-9 or in vitro lineage tracing10-13. These studies demonstrated that HPCs arise within the liver, engraft the parenchyma14 and may have some differentiation capacity but did not regenerate significant quantities of liver parenchyma11-13. Only if HPCs have regeneration capacity, are expandable in vitro and transplantable would they be a potential future therapeutic target. Many dietary and chemical models of injury have been exploited to investigate the biology of HPCs (refs 15-17). Experiments utilizing hepatocyte lineage tracing in mice have shown in various liver injury models that hepatocytes regenerate themselves without any significant contribution from HPCs (refs 18,19). This calls into question the nature and role of HPCs in liver injury and regeneration20. Further experiments in mice have shown that hepatocytes can change into a biliary ductular phenotype21,22 and then later re-differentiate into hepatocytes23.

In advanced human liver disease there is often widespread hepatocyte senescence, that is, an irreversible block to hepatocyte replication, indicated by p21 or p16 positivity. In this setting, ductular reactions develop; however, the functional role of putative HPCs in human liver disease is difficult to discern in the absence of lineage tracing24. The question arises as to whether mouse models of liver injury adequately reflect human disease. In the rat, complete suppression of hepatocyte proliferation can be achieved using chemical toxins, which provokes an extensive ductular/HPC response which is thought to regenerate parenchyma, although lineage tracing studies are required to formally prove this25. The transdifferentiation of hepatocytes into biliary ductules

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is damage dependent and negligible unless significant injury is induced. To model the human (and rat) situation, we have utilized a genetic means of inducing hepatocyte injury and senescence in adult mouse liver. We have exploited an AhCre system with an Mdm2
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RESULTS

Transgenic targeted hepatocellular injury as a model of whole-organ repair

To determine whether endogenous ductular cells give rise to hepatocytes, we analysed a lineage tracing system using the CDE (choline-deficient ethionine-supplemented) diet–recovery model (Supplementary Fig. 1a). To label biliary/ductular cells we used the Krt19CreERT2 LSL13Tomato mouse and found labelling to be strictly limited to ducts in the uninjured liver with zero hepatocyte labelling (Supplementary Fig. 1b). Following CDE–recovery there was a minor degree of tracing into hepatocytes (Supplementary Fig. 1b,c), indicating that in this model the ductular–hepatocyte regeneration pathway is insignificant. We found minimal hepatocyte senescence (Supplementary Fig. 1b), in contrast to advanced human diseases and rat models of HPC activation where high levels of hepatocyte senescence occur. To analyse liver regeneration in advanced injury we therefore studied a model of induced hepatocyte senescence in mouse. The activation and proliferation of HPCs in vivo requires both hepatocellular injury and inhibition of hepatocyte replication. To achieve this we utilized the AhCre transgenic line, which contains the rat Cyp1A1 promoter cloned upstream of Cre recombinase, and we combined this line with a transgenic Mdm2 line in which exons 5 and 6 are flanked with loxP sites (Mdm2
to target hepatocyte injury and senescence in adult mouse liver. We have exploited an AhCre system with an Mdm2

Widespread hepatocyte injury promotes a ductular reaction

Following βNF administration, ΔMdm2 mice developed jaundice after three days and required humane euthanasia by day 8 (Supplementary Table 1 and Supplementary Fig. 8). Serum markers for liver injury (aspartate aminotransferase, bilirubin, alkaline phosphatase and alanine transaminase) were all elevated and serum albumin levels fell, signifying injury and impaired synthetic function of the livers of ΔMdm2 mice (Fig. 1h–j and Supplementary Fig. 2e,f). This reduction in functionality is associated with hepatocyte necrosis by haematoxylin and eosin (H&E) staining, which shows clear disruption of the hepatic architecture as well as increased positivity of lactate dehydrogenase staining (Fig. 1k and Supplementary Fig. 2h). The levels of apoptosis in ΔMdm2 mice were determined through TUNEL staining, which was absent in uninduced AhCre+ Mdm2+/− mice; however, we found clear positive cells throughout the parenchyma in AhCre+ Mdm2+/− mice that had received βNF (Fig. 1l). Further to this, we found increased messenger RNA expression of the p53-dependent apoptotic gene Bax1 (Supplementary Fig. 2g) throughout the 8-day time course in ΔMdm2 mice.

Uninduced AhCre+ Mdm2+/− mice have normal hepatic architecture and do not express detectable p53 protein in hepatocytes (Fig. 1b). Two days following induction, and Cre activation, with 20, 40 or 80 mg kg−1 of βNF, rapid expression of p53 protein was observed in hepatocyte nuclei in a dose-dependent manner (Fig. 1b,c); however, following βNF administration to Mdm2+/− mice lacking the AhCre transgene, we found no expression of p53 protein, confirming that p53 expression is a consequence of Cre activity and Mdm2 loss (Fig. 1d). We also observed expression of p21 protein in hepatocytes following βNF administration (Fig. 1e) but not in Cre− animals (Fig. 1f). To validate Mdm2 recombination in this system following Cre activation, we isolated a highly purified population of hepatocytes and non-parenchymal cells (NPCs) two days following high-dose (80 mg kg−1) βNF administration. We evaluated the presence of both exon 5 (which would be lost following Cre-mediated recombination) and exon 3 (which would remain intact following Cre-mediated recombination), thereby offering an internal control. In hepatocytes there was a 96.8% reduction in the quantity of genomic Mdm2 exon 5 following recombination but only a 0.8% reduction in NPCs from the same cohort (Fig. 1g and Supplementary Fig. 2a–d) indicating that the AhCre transgene is highly specific in recombining ΔMdm2 in hepatocytes.

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Widespread hepatocyte injury promotes a ductular reaction

Following βNF administration we found small cells appearing in the livers of ΔMdm2 mice that did not upregulate p53. We examined the ductular reaction in ΔMdm2 livers to ascertain whether HPCs arise in this model. In the uninjured liver, panCK is expressed in the ductular reaction in the Krt19CreERT2 LSL13Tomato mouse and found labelling to be strictly limited to ducts in the uninjured liver with zero hepatocyte labelling (Supplementary Fig. 1b). Following CDE–recovery there was a minor degree of tracing into hepatocytes (Supplementary Fig. 1b,c), indicating that in this model the ductular–hepatocyte regeneration pathway is insignificant. We found minimal hepatocyte senescence (Supplementary Fig. 1b), in contrast to advanced human diseases and rat models of HPC activation where high levels of hepatocyte senescence occur. To analyse liver regeneration in advanced injury we therefore studied a model of induced hepatocyte senescence in mouse. The activation and proliferation of HPCs in vivo requires both hepatocellular injury and inhibition of hepatocyte replication. To achieve this we utilized the AhCre transgenic line, which contains the rat Cyp1A1 promoter cloned upstream of Cre recombinase, and we combined this line with a transgenic Mdm2 line in which exons 5 and 6 are flanked with loxP sites (Mdm2
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Mdm2+ Mdm2+/− mice were highly purified and sorted (FACS). Here we found an expansion of a cell population that at comparable levels. In line with this increase in HPC number, we observed a significant increase in mRNA expression of HPC markers EpCAM and CD24 (Fig. 2d and Supplementary Fig. 3d). In contrast, we found that the cell population that express EpCAM and CD24 are restricted to the biliary epithelium in the healthy liver (Fig. 2b,c and Supplementary Fig. 3a–c). Following AhCre induction, the migrating cords of HPCs also express the HPC markers EpCAM and CD24 (Fig. 3a and Supplementary Fig. 3d). The extent of the ductular reaction in the ΔMdm2 was significantly greater than in the CDE diet (Supplementary Fig. 3g). In the ΔMdm2 livers, Sox9-positive...
HPCs did not express CYP2D6 (Fig. 2i). Furthermore, HPCs were not affected by the Cre system in response to the βNF induction, as they lacked upregulation of p53 (Fig. 2j), and are proliferative following βNF administration and recombination of the Mdm2loxP locus in hepatocytes (Fig. 2k). Proliferative bromodeoxyuridine (BrdU)-positive HPCs were often found closely association with
BrdU-positive hepatocytes 8 days following AhCre induction, suggesting that hepatocytes arise from BrdU-positive HPCs in this model. Importantly, we also found that 2 days after induction there are no BrdU-positive hepatocytes adjacent to BrdU-positive HPCs, suggesting that proliferation of HPCs themselves does not lead to hepatocyte proliferation (Supplementary Fig. 3h).

**Large-scale hepatocyte replacement can arise from endogenous HPCs**

The AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> model results in massive hepatocyte injury, where more than 96% of hepatocytes accumulate p53, and activates a florid HPC response. To investigate how significant the HPC contribution is to regeneration we sought to induce the Mdm2 transgene and follow progenitor cells out to 6 months, to evaluate whether they can reconstitute the liver efficiently.

To avoid the early mortality observed in AhCre+ Mdm<sup>flox/fox</sup> mice, we examined the long-term effects of Mdm2 recombination in AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> mice. AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> mice had a somatic heterozygotic deficiency of the Mdm2 gene, but are phenotypically normal until the second Mdm2 allele is lost. Recombination was as efficient in the AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> as in AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> counterparts (Supplementary Table 1 and Supplementary Fig. 8). In

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**Figure 2** Hepatocyte Mdm2 loss results in rapid activation of HPCs. (a) Immunohistochemistry for HPCs (panCK, brown) in uninduced AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> mice. (b) Immunohistochemistry for HPCs (panCK, brown) in ΔMdm2 mice 8 days following induction with IFN. (c) Quantification of mean number of panCK<sup>+</sup> cells per field over the 8-day time course following induction with IFN (mean ± s.e.m., Kruskal–Wallis test; P=0.0016; n=7, 3, 3, 4 and 5 mice for days 0, 2, 3, 5 and 8 respectively). (d) Immunohistochemistry for EpCAM<sup>+</sup>CD24<sup>+</sup> HPCs (EpCAM, green; CD24, red) in ΔMdm2 mice 8 days following induction with IFN. White arrows show EpCAM<sup>+</sup>CD24<sup>+</sup> HPCs. (e) Immunohistochemistry for EpCAM<sup>+</sup>CD24<sup>+</sup> HPCs (EpCAM, green; CD24, red) in uninduced AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> mice. (f) FACS analysis of EpCAM<sup>+</sup>CD24<sup>+</sup>CD133<sup>+</sup> HPCs in both 12-day CDE-treated mice and IFN-induced AhCre<sup>+</sup> Mdm<sup>flox/fox</sup> mice. (g,h) mRNA expression of EpCAM and Dlk1 over the experimental time course following induction with IFN (mean ± s.e.m., (g) P=0.024 on day 6, P=0.036 on day 8, (h) P=0.036 on day 8, one-way ANOVA with Bonferroni correction; n=3, 3, 6 and 5, and 3, 3, 3 and 3 mice for days 2, 3, 5 and 8 for experimental and controls respectively). (i) Immunohistochemistry of 4 days post ΔMdm2 showing hepatocytes, CYP2D6 (red) and HPCs, Sox9 (green). (j) Immunohistochemistry of day 8 ΔMdm2 showing hepatocytes (CYP2D6, blue), HPCs (Sox9, red) and p53 (green). White arrow: CYP2D6<sup>+</sup>p53<sup>+</sup> hepatocytes. Yellow arrow: Sox9<sup>+</sup> HPC adjacent to p53<sup>+</sup> hepatocytes. (k) Immunohistochemistry of HPC proliferation, HPCs (panCK, green) and proliferation (BrdU, red). Arrows mark BrdU-labelled HPCs. BrdU-labelled hepatocytes are associated with HPCs. The images shown here are representative for 2 experiments with 3–5 mice in each group per experiment. Scale bars, 50 μm.
AhCre<sup>+</sup> Mdm2<sup>flox/−</sup> mice, the onset of liver injury and clinical signs of acute disease, demonstrated through necrosis and apoptosis by H&E and TUNEL staining (Fig. 3a,b), together with tachypnoea, hunching, prostration and bilirubinuria, occur between 14 and 21 days following AhCre activation, all of which are delayed in the Mdm2<sup>flox/−</sup> compared with the Mdm2<sup>flox/flox</sup> mice given the same induction regime. Concurrently, HPC activation was more gradual than in the AhCre<sup>+</sup> Mdm2<sup>flox/−</sup> counterparts and arose from day 14 post Mdm2 deletion (Fig. 3c). The most likely explanation for this delay is that compensatory changes occur in the hepatocytes that already lack one allele, so when the second allele is removed the effect is less pronounced. A similar effect of Mdm2 haploinsufficiency has been described in the context of tumour development<sup>30</sup>. Furthermore, no evidence of stable, nuclear p53 could be found in HPCs arising in the AhCre<sup>+</sup> Mdm2<sup>flox/−</sup> mice, indicating that this long-term model faithfully recapitulates what we see in Mdm2<sup>flox/flox</sup> mice (Fig. 3d,e).

In AhCre<sup>+</sup> Mdm2<sup>flox/−</sup> mice following βNF administration, a large proportion developed liver damage and then subsequently made full recoveries with dissipation of liver injury and the ductular reactions (Fig. 3f) by 6 months. As the AhCre is highly efficient in the liver we used the presence of nuclear p53 in hepatocytes to ascertain the HPC-mediated hepatocellular regeneration. As anticipated, two days following βNF we found that most hepatocytes expressed the p53 protein (98.9%; Fig. 4a,b). In addition to the high level of p53 in the liver, almost all hepatocytes (99.96%) express the senescence marker p21, suggesting that these cells enter either direct or indirect senescence 2 days following βNF (Fig. 4c,d). At 19 days following induction, p53 staining in hepatocytes was more heterogeneous, with 93.5% of hepatocytes still expressing p53 (Fig. 4a,b). Six months
following induction of Cre recombinase, hepatocytes were largely p53 negative (only 0.14% of hepatocytes were positive). The sequential dilution of p53 positivity from the hepatocyte population reflects the progressive replacement of Mdm2-negative hepatocytes from the parenchyma by HPC-derived (Mdm2 intact, p53 low) hepatocytes.

To confirm that this loss of p53 positivity was not due to downregulation of p53 by other mechanisms, we used the AhCre+ Mdm2flox−/− mice crossed onto a silenced R26RLacZ reporter. Following recombination, LacZ expression could be detected throughout the intestine and liver. Loss of reporter was seen over 6 months, indicating that replacement of the recombined epithelium occurs from a non-recombined source (Fig. 4e). Following recovery of AhCre+ Mdm2flox−/− mice, no animals developed liver tumours or notable fibrosis (Fig. 4f).

**Endogenous HPCs are required for adult liver regeneration following Mdm2 loss**

In our AhCre+ Mdm2flox−/− mouse model we wanted to evaluate the kinetics of cell replacement from p53-negative HPCs. Proliferating hepatocytes, positive for Ki67, were arranged in cord-like formations, with each cord of dividing hepatocytes being in contact with the distal tips of the ductular reaction (Fig. 5a). This evidence, although indirect, suggested a role for HPCs in either supporting hepatocyte proliferation or differentiating into hepatocytes.

Fn14 has been identified as a critical receptor for mitogenic stimulation of HPCs (refs 31–34). To confirm that the regeneration we see in the AhCre Mdm2 system is a direct result of HPC activation and differentiation, and not due to a small number of hepatocytes that escape Mdm2 recombination, we crossed the AhCre+ Mdm2flox−/− mouse onto an F14 null (F14KO) background34. We induced the AhCre+ Mdm2flox−/−F14KO mice and tracked the fate of these animals over 30 days (Fig. 5b). In our AhCre+ Mdm2flox−/− model where F14 is intact we detect upregulation of F14 mRNA following induction with βNF over four days (Fig. 5c). Following Cre induction, F14KO ΔMdm2 animals demonstrated a significantly reduced survival, and all had to be euthanized within 10 days (Fig. 5d), unlike F14WT ΔMdm2 mice in which 80% of animals survived to day 30. The induction of damage and repair on the background of F14 loss
Figure 5 Fn14/TWEAK-regulated HPCs are necessary for liver regeneration following hepatocyte Mdm2 deletion. (a) Immunohistochemistry for proliferation (Ki67) demarcated in white and HPCs (panCK) demarcated in red 19 days following ΔMdm2 in AhCre\(^+\)Mdm2\(^{floxed/}\) mice. (b) Schematic representing the experimental time course using the Fn14\(^{KO}\) AhCre\(^+\)Mdm2\(^{floxed/}\) model with Fn14\(^{KO}\) AhCre\(^+\)Mdm2\(^{floxed/}\) controls 4 days following 80 mg kg\(^{-1}\) βNF administration. (g) Active nuclear NFκB p65-expressing panCK\(^+\) HPCs observed in Fn14\(^{+}\) mice (arrows) but not in HPCs from Fn14\(^{-}\) mice (arrowheads). Sox9\(^+\)/CYP2D6\(^+\) co-staining association of Sox9\(^+\)/CYP2D6\(^{low}\) HPCs (open arrows) with Sox9\(^+\)/CYP2D6\(^{intermediate}\) cells with intermediate morphology (arrowheads) and Sox9\(^+\)/CYP2D6\(^+\) hepatocytes (arrows) in each experimental group. The images shown here are representative for 3–7 mice in each group per experiment. Scale bars, 50 µm.

HPC number in a WT uninjured liver (mean ± s.e.m., Mann–Whitney test; \(P = 0.029\); \(n = 3\) versus 4 mice). (f) Representative images of panCK\(^+\) and Sox9\(^+\) HPCs (insets high power) in the Fn14\(^{+}\) model with Fn14\(^{KO}\) controls 4 days following 80 mg kg\(^{-1}\) βNF administration. (g) Active nuclear NFκB p65-expressing panCK\(^+\) HPCs observed in Fn14\(^{+}\) mice (arrows) but not in HPCs from Fn14\(^{-}\) mice (arrowheads). Sox9\(^+\)/CYP2D6\(^+\) co-staining association of Sox9\(^+\)/CYP2D6\(^{low}\) HPCs (open arrows) with Sox9\(^+\)/CYP2D6\(^{intermediate}\) cells with intermediate morphology (arrowheads) and Sox9\(^+\)/CYP2D6\(^+\) hepatocytes (arrows) in each experimental group. The images shown here are representative for 3–7 mice in each group per experiment. Scale bars, 50 µm.

corresponded with failed ductular expansion (Fig. 5e,f), indicating that in the AhCre\(^+\)Mdm2\(^{floxed/}\) mouse, HPCs are critical for hepatic regeneration. HPCs had a corresponding reduction in NFκB activity (Fig. 5g), a downstream mediator of TWEAK/Fn14 signalling.\(^{33}\) We treated ΔMdm2 mice (which are WT for the tweak receptor Fn14) throughout the onset of injury with 4 doses of intravenous TWEAK (Fig. 6a). At 4 days following activation of Cre, animals that had been given TWEAK had significantly increased numbers of ductular cells versus vehicle alone (Fig. 6b,c). TWEAK-treated ΔMdm2 mice also had a higher frequency of p53-negative hepatocytes adjacent to ductular cords (Fig. 6d). To confirm that the effect of TWEAK is on ducts instead of hepatocytes, we isolated hepatocytes from βNF-induced animals and did not observe upregulation of Fn14 in hepatocytes after injury (Fig. 6e). These data suggest that following loss of Mdm2 in
hepatocytes. Large-scale parenchymal injury prompts ductular expansion in a TWEAK/Fn14-dependent manner. This provides evidence of the beneficial effect of HPC to liver regeneration when hepatocyte senescence prevents normal hepatocyte regeneration.

**CD45<sup>−</sup>CD31<sup>−</sup>Ter119<sup>−</sup>EpCAM<sup>+</sup>CD24<sup>+</sup>CD133<sup>+</sup> marks a population of bipotent HPCs**

The *AhCre<sup>+</sup>Mdm2<sup>flx/flx</sup>* model demonstrates the efficacy of adult HPCs to regenerate the adult liver. In human disease, cells analogous to the murine HPCs arise in response to biological or chemical damage<sup>24,35</sup> (not typically genetic alteration), so to assess whether *ex vivo* HPCs can also significantly repopulate the adult liver we used a chemical model of hepatocellular disease, the murine CDE diet, previously employed to trace the fate of HPCs *in vivo<sup>21,36,37</sup>*. Isolation of these HPCs by FACS has been variable<sup>8,36,37</sup>; therefore, we used HPC markers described in the literature, in combination, to identify the most potent HPC sub-population.

The CDE diet resulted in ductular reactions arising from the terminal ducts of the portal tracts compared with control diet (Fig. 7a,b). Within the panCK-expressing ductular reactions, we observe a subpopulation of HPCs that express the surface markers EpCAM and CD24; whereas EpCAM is very specific for ductular cells, CD24 labels both HPCs and a population of hepatocytes *in vivo* (Fig. 7c and Supplementary Fig. 3d). To exclude these CD24-positive hepatocytes, and to ascertain whether these CD24<sup>−</sup>EpCAM<sup>+</sup> cells were *bona fide* HPCs, we used FACS to isolate multiple candidate HPC populations. We negatively selected for haematopoietic (CD45), endothelial (CD31) and erythroid (Ter119) lineages and then positively selected for EpCAM, CD24 and CD133 (Fig. 7d), thereby generating three candidate HPC populations.

**Figure 6** TWEAK enhances the ductular reaction through activation of HPCs. (a) Schematic representing the experimental time course using the *AhCre<sup>+</sup>Mdm2<sup>flx/flx</sup>* mice that receive intravenous injection of TWEAK. (b) Quantification of number of panCK<sup>+</sup> HPCs in ΔMdm2 control versus ΔMdm2 given recombinant TWEAK (mean ± s.e.m., Mann–Whitney test; *P* = 0.029; *n* = 3–4 mice per group). (c) panCK staining in ΔMdm2 control mice versus ΔMdm2 mice given recombinant TWEAK. (d) p53 immunohistochemistry and analysis of portal tracts of both ΔMdm2 controls and mice treated with repeated injection of i.v. TWEAK, showing p53<sup>−</sup>-cells with hepatocyte-like morphology adjacent to portal tracts and areas of ductular expansion 4 days following induction (80 mg kg<sup>−1</sup> iNF). Arrows show p53<sup>−</sup> HPCs and arrowheads show p53<sup>−</sup> hepatocytes. (e) mRNA expression of Fn14 from isolated HPCs and, hepatocytes isolated from wild-type mice, and hepatocytes isolated from ΔMdm2 mice (mean ± s.e.m., one-way ANOVA; *P* = 0.0129; *n* = 3 mice). The images shown here are representative for 3–4 mice. Scale bars, 50 µm.
Figure 7  
**In vitro** expanded HPCs are genetically and phenotypically stable. (a,b) Immunohistochemistry for HPCs (panCK) in uninjured liver (a) and mice treated for 12 days with CDE diet (b). (c) Immunohistochemistry on serial sections for HPCs (panCK) and EpCAM (green), CD24 (red) and DAPI (blue). Arrows show EpCAM+CD24+ HPCs. (d) FACS gating strategy to isolate 7-AAD+ and DAPI (blue). Arrows show EpCAM+CD24+ HPCs from uninjured and CDE-injured liver. The data shown here are representative images for 10 mice. (e) Histogram representing the percentage of EpCAM+CD24+CD133−, EpCAM+CD24+CD133+ and EpCAM+CD24−CD133− populations in the NPC fraction of healthy or CDE-treated livers (mean ± s.e.m., Mann–Whitney test; P = 0.0025; n = 4 mice each group). (f) Colony-forming efficiency of EpCAM+CD24+CD133−, EpCAM+CD24+CD133+ and EpCAM+CD24−CD133− populations in vitro (mean ± s.e.m., one-way ANOVA; P = 0.032, and 0.002 respectively; n = 5 biological replicates per group). These data are representative of 3 individual experiments. (g) Phase-contrast images of the colonies formed by the EpCAM+CD24+CD133−, EpCAM+CD24+CD133+ and EpCAM+CD24−CD133− populations. (h) Frequency of chromosome number in metaphase spreads from **in vitro**-expanded HPCs. Inset, a representative example of a normal karyotype from **in vitro**-expanded cdHPCs. (i) Immunocytochemistry of HPC markers in **in vitro**-expanded HPCs. (j) Schematic representation of the experiment to determine the origin of cdHPCs. (k) Immunohistochemistry on healthy, tamoxifen-induced Krt19CreERT/LSL/TdTomato mice for recombined HPC, CK19 (green), TdTomato (red) and DAPI (blue). (l) Percentage of the EpCAM+CD24+CD133− population expressing TdTomato in uninjured Krt19CreERT/LSL/TdTomato mice, Krt19CreERT/LSL/TdTomato mice treated with CDE diet, and Krt19CreERT/LSL/TdTomato mice treated with CDE diet followed by 14 days of normal diet (mean ± s.e.m., one-way ANOVA; P < 0.05 (NS, not significant), n = 5 mice). (m) Merged phase-contrast and fluorescent image of isolated EpCAM+CD24+CD133−/TdTomato+ cells from Krt19CreERT/LSL/TdTomato mice receiving CDE diet. Data are represented as mean ± s.e.m. n ≥ 3 each group. **In vitro** data represent three independent experiments. Scale bars, 50 µm.

Table: Chromosome number in metaphase spreads from **in vitro**-expanded HPCs.

| Chromosome number | Frequency of chromosome number (percentage) |
|-------------------|--------------------------------------------|
| 4                  | 50.5%                                       |
| 5                  | 62.4%                                       |
| 6                  | 77.4%                                       |
| 7                  | 86.6%                                       |
| 8                  | 95.5%                                       |

Note: NS, not significant; P = 0.0025; n = 4 mice each group.

The data represent three independent experiments.

Besides an increase in total NPC population, all three populations were enriched in the NPC fraction of livers from mice fed the CDE diet compared with the control diet (Fig. 7g). These three populations were plated at clonal density to assess their colony-forming potential. EpCAM+CD24+CD133− and EpCAM+CD24−CD133− populations...
and EpCAM⁺CD24⁻CD133⁻ populations formed infrequent, small colonies with mesenchymal characteristics, whereas the EpCAM⁺CD24⁺CD133⁺ population generated frequent colonies of packed epithelial cells, from herein known as clonal-density-derived HPCs (cdHPCs; Fig. 7f,g).

**The defined population of HPCs can be expanded in vitro**

Over six weeks, cdHPCs expand and proliferate in vitro. Historically, expanding HPCs has been hampered by cells differentiating following replating; we therefore adapted a new method for passaging these cells where, rather than being passaged as a single-cell suspension, they are detached using highly diluted trypsin and lifted and replated as colonies, ensuring that cell–cell contact is maintained. Using this method we found that the viability and phenotypic stability of passed cdHPCs increased (Supplementary Fig. 4a). In our assay, only the cdHPC population can be expanded whilst maintaining an epithelial morphology; the other two populations failed to maintain any epithelial phenotype. The expanded cdHPC population can be maintained long term in vitro (more than 30 passages and up to a year) and remains chromosomally stable having a normal karyotype (Fig. 7h). The in vitro-expanded cdHPCs maintained the co-expression of HPC markers EpCAM, CD24 and CD133 (Supplementary Fig. 4b). Expanded cdHPCs highly express HPC-related genes relative to housekeeping gene compared with hepatocytes (Supplementary Fig. 4c,d). Expanded cdHPCs maintained their HPC characteristics and were positive for a suite of markers (Sox9, CD44, Fn14, OPN1 and panCK) which have been used to define HPCs in vivo and the expression of these markers was maintained after 30 passages (Fig. 7i and Supplementary Fig. 4e,f). We did not observe a significant change in the expression of most HPC-related genes such as EpCAM, Krt19, Spp1 and Sox9, or Alb after long-term passaging, although Lgr5 expression increased (Supplementary Fig. 4e). After in vitro expansion, mean cell size reduced after 10 passages; however, no significant changes in elongation assessed by cell roundness, or cell width to length ratio were seen (Supplementary Fig. 4g). We calculated that in our in vitro expansion system each cell has divided around 32 times at passage 10 (Supplementary Fig. 4h). cdHPCs did not express endothelial (CD31), haematopoietic (CD45) or mesenchymal (GFAP and desmin) markers (Supplementary Fig. 5a,b). To ascertain the bipotentiality of cdHPCs we differentiated them towards biliary or hepatic lineages. Following three-dimensional culture of cdHPCs in Matrigel, we observed cells forming tubules, which had a branched morphology and lumen (Supplementary Fig. 5c) and expressed the hepatic markers albumin (Supplementary Fig. 5d). Murine albumin increased at the transcript level and as a protein in the culture medium (Supplementary Fig. 5e, upper histograms). Concurrent with this upregulation, we found loss of the biliary- and progenitor-associated genes Krt19, Epcam, Dlk1 and Afp and upregulation of the hepatocyte transcription factor Hnf1α (Supplementary Fig. 5e, lower histograms). When we reanalysed this population for EpCAM and CD24, we identified three populations: EpCAM⁺CD24⁻, EpCAM⁻CD24⁺ and EpCAM⁺CD24⁺. To further refine where in cdHPCs the most potent progenitor cell resides, we investigated the self-renewal ability of these cdHPC populations using a secondary clone assay. EpCAM⁺CD24⁻, EpCAM⁻CD24⁺ and EpCAM⁺CD24⁺ were sorted and plated at clonal density. EpCAM⁺ clones were capable of generating colonies; however, this was significantly impaired in EpCAM⁻ cdHPCs, regardless of CD24 expression status (Supplementary Fig. 6a–c).

To confirm the biliary/ductular origin of the cdHPCs, we isolated cdHPCs from tamoxifen-induced Krt19CreERT/LSLtdTomato mice without liver injury, during CDE injury, and 2 weeks after CDE injury (Fig. 7j). The recombination efficiency of Krt19-expressing cells after tamoxifen administration in the Krt19CreERT/LSLtdTomato mice was 62% (Fig. 7k). Zero Tomato expression was observed in the hepatocytes in any animal (Supplementary Fig. 1a). Likewise, the cdHPCs from tamoxifen-induced Krt19CreERT/LSLtdTomato mice without liver injury were 70% Tomato⁺ demonstrating their ductular origin. This percentage of TdTomato-expressing cdHPCs remained constant before, during CDE injury, and after recovery from CDE injury (Fig. 7l,m), arguing against a significant non-ductular contribution to this population.

**In vitro-expanded HPCs can repopulate the liver after hepatocellular injury**

To ascertain the in vivo liver repopulating potency of cdHPCs, we performed transplantation studies using cdHPCs. cdHPCs were transfected with a plasmid expressing CAG–GFP and stably transfected clones selected (Fig. 8a). GFP–cdHPCs (5 × 10⁶) were transplanted intrasplenically into the AhCre⁺Mdm2lox/lox mice that had previously received a low dose of βNF (10 mg kg⁻¹) to induce hepatocellular injury. Following transplantation, recipient AhCre⁺Mdm2lox/lox mice were given repeated doses of βNF (20 mg kg⁻¹) every 10 days to progressively ablate endogenous Mdm2lox/lox hepatocytes (Fig. 8a and Supplementary Table 1). Three months following transplantation, large patches of GFP⁺ cells were observed in the transplanted liver compared with controls (Fig. 8b). GFP-positive cells in the parenchyma were large and morphologically consistent with hepatocytes (Fig. 8c and Supplementary Fig. 7a). Transplanted animals had significantly improved liver histology with stronger glycogen staining (Fig. 8d top and middle panel), less liver scarring, (Fig. 8d bottom panel and Fig. 8e) and increased serum albumin levels compared with controls (Fig. 8f). The GFP-expressing, cdHPC-derived cells co-expressed the hepatocyte markers HNF4α and CYP2D6 and were found in patches adjacent to host GFP-negative hepatocytes (Fig. 8g and Supplementary Fig. 7b). Small GFP-positive ductular cells were seen that expressed panCK and SOX9 (biliary markers), indicating bi-lineage differentiation of transplanted cdHPCs in vivo (Fig. 8h). These GFP⁺ cells were found in both bile ducts and cords of ductular cells alongside host ductular cells (Supplementary Fig. 7b). cdHPC hepatocytes were proliferating (Ki67⁺) in the recipient liver at 3 months following transplantation, whereas there was little proliferation in the endogenous hepatocytes of AhCre⁺Mdm2lox/lox recipient mice. Concurrently, more senescent cells in the non-transplanted group were observed with p21 staining (Supplementary Fig. 7c).
Figure 8 Relationship between activated HPCs and hepatocytes. (a) A schematic representation of the AhCre<sup>+</sup> Mdm2<sup>flox/fox</sup> mice as a transplant recipient model of GFP-expressing HPCs. Expanded HPCs stably transfected with a CAG-GFP-expressing vector, (left) photomicrograph phase contrast, (right) GFP fluorescence. (b) Low-power epifluorescence of sham transplanted liver, ubiquitous CAG-GFP-expressing liver and ΔMdm2 liver with CAG-GFP-expressing transplanted in vitro-expanded HPCs. (c) Immunohistochemistry for GFP in non-transplanted ΔMdm2 controls versus ΔMdm2 transplanted with CAG-GFP-expressing HPCs. Black arrows denote GFP-expressing hepatocytes and red arrow GFP-expressing duct. Dot-plot shows quantification of GFP in transplanted versus non-transplanted liver (mean ± s.d., Student’s t-test; P = 0.0019; n = 17 fields at ×200 magnification). (d) Histochemical analysis of non-transplanted AhCre<sup>+</sup> Mdm2<sup>flox/fox</sup> mice and cdHPC-transplanted AhCre<sup>+</sup> Mdm2<sup>flox/fox</sup> mice.

**DISCUSSION**

The liver regenerates through hepatocyte proliferation following moderate liver injury, and hepatocyte cell therapy is a potential alternative to liver transplantation<sup>10,41</sup>. Liver regeneration following chronic or severe liver injury is less well defined. HPCs are activated during chronic liver disease when hepatocyte proliferation is impaired<sup>15,42,43</sup>; however, whether these HPCs regenerate parenchyma is controversial<sup>11,21,44,45</sup>. Using targeted deletion of Mdm2 in mouse hepatocytes, we have demonstrated that HPC activation is sufficient to regenerate a large proportion of the liver parenchyma. In our model, on Cre activation most hepatocytes (98.9%) accumulate p53, inducing senescence. To address the possibility that the 1.1% of unrecombined hepatocytes may regenerate the liver, we investigated the degree of hepatocyte senescence by p21 staining. Although the proportion of p53-high hepatocytes was 98.9%, almost all hepatocytes (99.96%) were p21 positive, possibly reflecting either under-detection of p53 by immunohistochemistry or the occurrence of spreading senescence as previously reported in vivo and in vitro<sup>16-48</sup>. The proliferation of p21-positive hepatocytes is irreversibly inhibited<sup>49</sup> making it unlikely that p21-positive hepatocytes contribute to regeneration. Furthermore, proliferating hepatocytes were located at the distal tips of the HPC-containing ductular reactions following BNF induction, suggesting that HPCs contribute to the formation of new p21-negative hepatocytes that are capable of proliferation.

To further investigate the importance of HPC activation, we used a model deficient in Fn14, a receptor for the HPC mitogen TWEAK (refs 33,34). Mice with Fn14 knockout do not mount an appropriate ductular reaction, and have increased mortality. Administration of TWEAK enhanced the ductular reaction in AhCre<sup>+</sup> Mdm2<sup>flox/fox</sup> mice following liver injury. These findings suggest that the TWEAK/Fn14-mediated ductular reaction is required to adequately regenerate liver parenchyma following such damage.

It has been shown that EpCAM<sup>+</sup> cells isolated from human liver can be expanded as organoids in vitro<sup>50</sup>. Whether these cells can...
regenerate large amounts of liver parenchyma will be important for their development as a human cell therapy. Here, we demonstrate that large-scale therapeutic liver repopulation can be achieved by transplanted HPCs.

CD45−CD31−Ter119−EpCAM+CD24+CD133+ HPCs have a high colony-forming capacity and are of biliary origin. Using the Krt19CreERT; LSL-TdT mice,10 lineage tracing model, we found that the degree of hepatocyte formation from ductular cells in the CDE diet and recovery model is limited.13,21,45, we speculate that this is due to the mild liver injury and lack of hepatocyte senescence, compared with advanced human liver disease.15 This lack of hepatocyte senescence in the CDE model thereby allows hepatocytes to regenerate themselves without recourse to a second tier of regeneration.

Although previous studies have isolated putative HPCs using FACS isolation, these techniques are based on single selection markers8,37 or did not completely exclude other NPC populations.32,52 We investigated multiple potential HPC populations by using a highly stringent FACS gating strategy without the need for a transgenic reporter. This is important as HPCs share markers with cholangiocytes and a defined selection strategy is required to reduce impurity if this is going to be translated into human studies. We have overcome the difficulty of expanding HPCs in vitro in monolayer conditions whilst maintaining their karyotypic and phenotypic stability. The CD45−CD31−Ter119−EpCAM+CD24+CD133+ HPC population is extremely rare in healthy liver, making their isolation from healthy tissue logistically challenging. Furthermore, the liver repopulating capacity of these cells in a healthy liver is likely to be insignificant—previous lineage tracing studies have shown no contribution from HPCs to hepatocytes in healthy liver9,11 and hepatocytes are the primary source of regeneration in normal liver homeostasis.10 Overall, our data suggest the existence of a facultative and functionally significant HPC population that does not contribute to homeostatic repair, but is activated in severe liver injury where hepatocyte senescence is widespread. We have used the term HPC throughout the data confirming genuine progenitor characteristics of these cells within the ductular reaction; however, in the absence of this functional evidence the term ductular cell may be more accurate.

So far, the repopulation capacity of transplanted HPCs has been limited and their ability to improve the structure of chronically damaged liver unproven. Using repeated rounds of hepatocyte senescence and injury in the AhCre+ Mdm2+ mice combined with HPC transplantation we found that the progeny of the transplanted HPCs can proliferate long term and regenerate large areas of normal parenchyma. The transplanted HPCs demonstrated bi-lineage differentiation into large numbers of hepatocytes and cholangiocytes in vivo causing significant structural and functional improvement of the damaged liver, with reduced scarring and increased serum albumin in transplant recipients. Our characterization of a defined population of HPCs indicates their biological significance and future therapeutic potential for the treatment of human liver disease.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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

Animal models. The animals used in this study were all on a C57Bl/6 background aged within 8–12 weeks old at the start of the experiments; both male and female mice were used. Animals were housed in a specific pathogen-free environment, with standard laboratory conditions (12 h day/night cycle) and access to food and water ad libitum. All animal experiments were carried out under procedural guidelines, severity protocols and with ethical permission from the Animal Welfare and Ethical Review Body (AWERB) and the Home Office (UK). Power calculations were not routinely performed; however, animal numbers were chosen to reflect the expected magnitude of response taking into account the variability observed in previous experiments. AhCre mice were crossed with Mdm2flox/flox, Mdm2−/−, and Mdm2+/-; mice to generate AhCre; Mdm2−/− mice and AhCre; Mdm2+/- mice. Controls. AhCre; Mdm2flox/flox or Mdm2−/− mice were also crossed with F1N4 (provided by Biogen Idec). Genotyping was performed for AhCre using 5′-CTGCTAAGTCAGGCGATC-3′ (forward) and 5′-ATTGCCCTGTTCATCATAC-3′ (reverse) primers and Mdm2 as previously described. AhCre was induced by intra-peritoneal (i.p.) injection of β-naphthoflavone (BNF; Sigma) at 20–80 mg kg−1, prepared previously under sterile conditions at 1% (v/v) in corn oil. K19Cre was induced by 3 individual i.p. injections of tamoxifen (20 mg ml−1) for 1 min to separate them from a non-Cre capsule disrupted to yield a cell suspension that was collected in Liver Perfusion Medium. NPCs were retained from the supernatant following the initial centrifugation step. Following red cell lysis (3 min in 160 mM NH4Cl) NPCs were retained from the supernatant following the initial centrifugation step. Following red cell lysis (3 min in 160 mM NH4Cl) NPCs were retained from the supernatant following the initial centrifugation step.

Coloncy-forming assay. Cells were plated at clonal density (250 cells cm−2) on collagen-coated plates with culture medium. The number of colonies comprised of more than 50 cells was counted two weeks after plating. Number of colonies formed/number of cells plated = colony-forming efficiency.

Expansion of HPCs. Sorted HPCs were cultured in HPC Expansion Medium: Williams’ Medium E (Gibco) containing 10% FCS, 17.6 mM NaHCO3, 20 mM HEPES pH 7.5, 10 mM nicotinamide, 1 mM sodium pyruvate, 1 × insulin, transferrin, selenium solution (ITS) (Gibco), 100 nM dexamethasone, 0.2 mM ascorbic acid, 14 mM glucose, 10 mM IL-6 (Peprotech), 10 nmol l−1 HGF (Peprotech), 10 nmol l−1 EGF (Sigma-Aldrich).

Cultured HPCs were washed with PBS. Cells were then incubated with diluted trypsin (0.25% trypsin no EDTA, 20% Knockout Serum Replacement, 1 mM CaCl2) at 37° C. Williams’ Medium E supplemented with 10% FCS was used to inhibit the reaction of trypsin. Cells were centrifuged at 300 g for 5 min; supernatant was then discarded. Cells were resuspended in culture medium and replated on collagen-coated plates.

Flow cytometry analysis. Cells were rinsed with PBS and then incubated with Cell Dissoication Medium (GIBCO) for 20 min at 37° C to prevent excessive cleavage of desired epitopes for detection. Cells were then incubated with anti-mouse CD16/32 antibody (eBiosciences) at 1:5 dilution for 10 min on ice. After rinsing with PBS, cells were resuspended in PBS + 2% FCS and incubated with antibodies (indicated in Supplementary Table 2) for 1 h at 4 °C. Cells were then washed with PBS + 2% FCS and then resuspended in PBS + 2% FCS for analysis or sorting. Stained samples were compared to unstained and isotype controls.

Immunohistochemistry. Paraaffin sections (3-μm-thick) were stained for the published HPC markers: panCK, A6 (provided by V. Factor; National Cancer Institute, Bethesda, Maryland, USA), Sox9, CD24, Dkki, EpCAM and Fn14 for the alternative antigens: BrdU (AB6326 Abcam), p53 (MS 104-P1, LabVision), Ki67 and CYP2D6 (gift from R. Wolfe, University of Dundee, UK); see Supplementary Table 2 for additional details. Cytosins and cells were fixed in cold methanol at 4 °C for 5 min before staining Species isotype (Santa Cruz) staining controls were routinely performed. Detection was performed with DAB (DAKO) followed by counterstaining with haematoxylin or alternatively with Alexa 488, 555 or 650 (A21206, A21434A21436E3S23355, and A21448 respectively; Invitrogen) with a DAPI-containing Vectashield mounting media (Vector). Apoptosis was assessed using the DeadEnd Colorimeter or Fluorometer (Alexa 488) TUNEL System (Promega no. TB199) according to the manufacturer’s instructions.

Microscopy and cell counting. Images were obtained on a Zeiss Axiovert 200 microscope using a Zeiss Axiocam MR camera. Cell counts were manually performed on blinded slides and more than 20 consecutive non-overlapping fields at ×200 or ×400 magnification. HPCs were defined as previously described and interlobular bile ducts were excluded from quantification. Confocal image analysis was performed using a Leica TCS-SP5 with the pinhole set to 1 airy unit. DAPI, Alexafluor 488 and 555 were detected using band paths of 415–480, 495–540 and 600–650 nm for 405, 488 and 543 nm lasers respectively.

Cell transplantation assay. AhCre Mdm2flox/flox mice were used as recipients for transplantation assays. To induce injury jFN (10 mg kg−1) was injected i.p. 4 days before transplant of 5 × 104 green fluorescent protein (GFP)-expressing cells suspended in 200 μL PBS delivered by intraperitoneal injection during laparotomy. The transplantation control group received 200 μL PBS alone. Recipient mice received intraperitoneal injections of 20 mg kg−1 jFN every 10 days after transplantation to induce persistent liver injury. Mice were euthanized and the liver was collected 12 weeks after cell transplantation.

In vitro differentiation of HPCs. Murine recombinant Wnt3a (100 ng ml−1; R&D systems) and 1% dimethylsulphoxide (DMSO) were added to HPCs in combination with culture medium when cultured HPCs were 70% confluent. The differentiation process lasted for 6 days; medium was changed once in 3 days. Medium was kept for enzyme-linked immunosorbent assay (ELISA) and cells were lysed for RNA isolation.

Formation of progenitor cell spheres. HPCs were trypsinized into a single-cell suspension by incubating with Cell Dissoication Medium (GIBCO) at 37 °C for 15 min and resuspended in 50 μl of undiluted Growth Factor Reduced Matrigel (BD) and plated on 24-well multwell plates (Corning). Each 50 μl of Matrigel that supplemented with previously published HPC expansion medium. A step-by-step protocol describing the isolation and expansion of HPCs can be found at Protocol Exchange®.

HPC isolation. NPCs were retained from the supernatant following the initial 135g centrifugation step. Following red cell lysis (3 min in 160 mM NH4Cl, 10 mM KHCO3, containing 0.01% EDTA), cells were re-suspended in Williams’ Medium E (Gibco) with 10% FCS and layered under an equal volume of 20% and 50% (v/v) Percoll (Sigma) in PBS, respectively. Following centrifugation at 1:400g for 20 min at 4 °C, the HPC-rich fraction lying between the 20% and 50% Percoll layers (enriched for non-parenchymal cells) was collected, washed twice and resuspended in PBS with 2% FCS for FACS staining procedure. NPCs collected as described above were resuspended in PBS + 2% FCS before incubation with anti-mouse CD16/32 antibody (eBiosciences) for 10 min. After a wash with cold PBS, cells were resuspended in PBS + 2% FCS and were incubated with EpCAM–APC-conjugated antibody (eBiosciences); CD24–PeCy7-conjugated antibody; CD133–FITC-conjugated antibody CD45–PE-conjugated antibody; CD31–PE-conjugated; Ter119–PE-conjugated antibody (Biolegend). Labelled cells were washed with PBS containing 2% FCS and analysed by FACS using a FACS Aria II (BD) with 488 nm and 640 nm lasers using a 100 μm nozzle. Haematopoietic cells (CD45+), erythroid cells (Ter119) and endothelial cells (CD31+) were excluded by gating. Immune cells (CD45+), HSCs (CD34+), and HPCs (CD34−/−) were selected from this gate.

89% of purified cells were positive for CD45 and HPC marker-CD34−/−; remaining 11% positively stained for CD45 and HPC marker-CD34+. To determine the purity of a cell population, FACS analysis of a single cell sample was used to exclude dead cells, 10 ng.ml−1 7-AAD (Biolegend) was added. Cells were analysed and sorted with a FACS Aria II (BD). Trigger pulse width was used to exclude cell doublets from analysis and collection. Sorted cells were plated on rat tail collagen I (Sigma)-coated plates
consisted of around 10,000 cells per spheroid was cultured. The plate was then left in the incubator at 37 °C for 10 min for the Matrigel to set. Culture medium was then added to cover the Matrigel sphere. Spheres were cultured for 7 days and then analysed by immunocytochemistry.

GFP transfection of progenitor cell line and derivation of stably transfected lines. Qiagen Effectene was used for stable transfection. In vitro-expanded progenitor cells (5 × 10^5) were plated overnight. One microgram of vector with a puromycin-resistant CAG–GFP was used to stably transfect cells with GFP, provided by S. Lowell (University of Edinburgh, UK). Transfected cells were cultured for 3 days and GFP expression was detected with fluorescence microscopy. Around 10% of transfected cells expressed GFP after 4 days. The selection of stably transfected cells was carried with medium supplemented with 3 μg ml^-1 puromycin for 14 days and colonies were picked with cloning rings and grown to generate clonally derived stably transfected lines. GFP expression of stably transfected lines was constantly monitored with fluorescence microscopy.

ELISA detection of albumin. Conditioned medium from cultured cells was stored at −80 °C for further analysis. Stored medium was thawed at room temperature and tested for albumin concentration using the Mouse Albumin ELISA Quantification Set following the manufacturer's protocol. A standard curve was generated using supplemented kits with different concentrations of albumin. Absorbance at 450 nm of was read using a spectrophotometer (SPECTROstar Omega).

Analysis of cell area, roundness, and ratio of width to length. Cell area, roundness, and ratio of width to length were analysed using the Operetta high-content analysis system (PerkinElmer). Data analysis was performed using the Columbus image data storage and analysis system.

Karyotyping of cells. Colecemic (Gibco) was added to the cultured cells for 18 h. Cells were then trypsinized, washed with PBS and resuspended with 75 mM KCl (Gibco) and incubated for 15 min at room temperature. Cells were then resuspended in fixatives (3:1 methanol and acetic acid). Cells were washed and spread on glass slides. Slides were mounted with DAPI fluoromount (Southern Biotech). The number of chromosomes was counted using fluorescence microscopy.

Real-time PCR and gene expression analysis. Genomic DNA (gDNA) was extracted from purified cell populations from whole liver using the DNA Blood Mini Kit (Qiagen). Total RNA was extracted from 30–50 mg tissue samples previously stored in RNAlater at −80 °C or cultured cells, using a combination of TRIzol reagent (Invitrogen) and Qiagen RNeasy Mini system according to the manufacturer's instructions (Qiagen). Reverse-transcription (including gDNA decontamination) and real-time PCR was performed using reagents and primers (Quantast and Quantitect respectively, Qiagen) on a Roche Lightcycler 480. Mdm2 integrity of gDNA was assessed using primers targeted to the floxed segment (forward: 5′-ACGAGAAGCAGCACACATTG-3′, reverse: 5′-TCGCCAGTGACACTCTCTAATG-3′). Data were analysed using the LightCycler system following normalization to the housekeeping gene peptidylprolyl isomerase A (Ppiu). All samples were run in triplicate.

Statistical analysis. Samples were randomized by a 'blinded' third party before being assessed separately by a 'blinded' assessor. Unblinding was performed immediately before final data analysis. Prism software (GraphPad Software) was used for all statistical analysis. Mean HPCs per ×200 magnification field from 30 fields for each mouse were compared. Data are presented as mean ± s.e.m. n refers to biological replicates. Normal distribution of data was determined using the D'Agostino and Pearson omnibus normality test. For parametric data, data significance was analysed using a two-tailed unpaired Student's t-test. In cases where more than two groups were being compared, then a one-way ANOVA was used. In instances where the n was too small to determine normal distribution or the data were non-parametric then a two-tailed Mann–Whitney U-test was used. F-tests were used to compare variances between groups.

Reproducibility of experiments. Routinely, qPCR experiments were performed in technical triplicates when possible. For representative images whole liver lobes were examined histologically in multiple biological replicates. For Figs 1b–f,k–l and 2a,b,d,e,i,j and Supplementary Figs 2h and 3a–c, the images are representative for 30 mice in total over a 3-year period. For Figs 2k,j and 4e and Supplementary Fig. 3a–d, the images are representative for 6 mice. For Figs 3a–f, 4a,c,f and 5a, the images are representative for 40 mice in total over a 1-year period. For Figs 5f,g and 6c,d, the images are representative for 7 mice. For Fig. 7g,l,m and Supplementary Figs 4a,f and 5b–d, the images are representative for 5 separate clonal cultures. For Fig. 7h, the image is representative of 50 cells. For Fig. 7a–d,k and Supplementary Fig. 1b,c, the images are representative for 10 mice. For Figs 8, 9a,d and Supplementary Figs 2a–c and 4a, the images are representative for 12 mice. For Supplementary Figs 5a and 6a,b, the images are representative for 7 separate clonal cultures.

Experiments in Figs 1g–j, 2c,g,h and 5c and Supplementary Figs 1a–c, 2a–h and 3e,f were repeated twice. For Figs 1g–j and 2c and Supplementary Fig. 2a–f these replicates consisted of repeated biological experiments with independent analysis; time points for analysis varied between experiments in 1h–j and 2c and Supplementary Fig. 2e,f. For Figs 2g,h and 5c and Supplementary Figs 2g and 3e,f, replicate experiments consisted of repeat analysis from biological samples from the level of RNA extraction. In Figs 7c,f and 8a–h and Supplementary Fig. 4b,c,e, 5e and 6c, flow cytometry and qPCR experiments were repeated 3 times; transplantation experiments are shown as representative from 3 experiments.

53. Goncalves, L. A., Vigario, A. M. & Penha-Goncalves, C. Improved isolation of murine hepatocytes for in vitro malaria liver stage studies. Malar. J. 169–176 (2007).
54. Lu, W. Y. et al. Isolation and expansion of the hepatic progenitor cell (HPC) population. Protoc. Exch. http://dx.doi.org/10.1038/protx.2015.051 (2015).
Supplementary Figure 1  Lineage tracing experiments to investigate the differentiation ability of HPCs.  (a) Schematic representation showing experimental design of lineage tracing experiments using the Krt19^CreERT^LSL^TdT^ Tomato mice. (b) Immunohistochemistry analysis for tdTomato and p21 on injured and uninjured tamoxifen induced Krt19^CreERT^LSL^TdT^ Tomato mice. (c) Immunohistochemistry for CYP2D (green), tdTomato (red), and DAPI (blue) on liver of tamoxifen induced Krt19^CreERT^LSL^TdT^ Tomato mice after CDE –recovery. The results shown are representative of 2 experiments with 5-8 mice each group. Scale bars = 50µm.
**Supplementary Figure 2** Administration of BNF induce hepatocyte damage. (a) Morphology by H&E and (b) expression of CYP2D6 by isolated purified hepatocytes following liver perfusion and digestion. Arrows denote examples of multinucleated hepatocytes. (c) Expression of nuclear p53 following extraction and purification of hepatocytes from AhCre⁺ Mdm2lox/lox mice (n = 3) 2 days following induction with 80mg/kg βNF compared to AhCre⁺ Mdm2lox/lox controls; arrow highlights low nuclear p53 expression. (d) Modified representation of Mdm2lox construct outlining primer targets for qPCR assessment of recombination efficiency. (e) Serum alkaline phosphatase (f) and ALT following induction in with 80mg/kg in AhCre⁺ Mdm2lox/lox animals (mean ± s.e.m, Kruskal Wallis Test, n = 3 mice each group, except day 8 where n=1 mouse due to cohort morbidity). (g) Expression of apoptosis associated p53-dependent gene Bax in whole liver over time following induction with 80mg/kg βNF in AhCre⁺ Mdm2lox/lox mice (mean ± s.e.m, One-way ANOVA.; n=3 mice each control time point and n = 3,3,5,6 for experimental time points). (h) Immunohistochemistry for lactate dehydrogenase of healthy mice, βNF induced AhCre⁺ Mdm2lox/lox controls and βNF induced AhCre⁺ Mdm2lox/lox mice. Representative images shown are representative of 2 experiments with 12 mice in total. Scale bars = 50 µm.
Supplementary Figure 3 Activation of ductular reaction following hepatocyte damage. Detection of (a) EpCAM (b) DLK1 (c) A6 (inset, AhCre control) expressing cells following ΔMdm2 in hepatocytes. (d) Immunohistochemistry for CD24 (red), EpCAM (green), DAPI (blue) on CDE treated and βNF induced AhCre+Mdm2^flox/flox mice. Representative images are shown are representative of 3 experiments with 5-8 mice each group. (e) Ck19 expression in the whole liver of the induced Mdm2flox/flox mice versus uninduced control over time (mean ± s.e.m, One-way ANOVA with Bonferroni correction. P=0.0058 day 8; n=3 mice each control time point and n = 3,3,6,5 for experimental time points, repeated twice). (f) Ascl2 expression of Mdm2^flox/flox mice over time following induction (mean ± s.e.m, One-way ANOVA with Bonferroni correction. P=0.05; n=3 mice, repeated twice). (g) Quantitative comparison of the panCK positive cells between the uninduced control, Mdm2^flox/flox, and the choline deficient ethionine supplemented diet (CDE) model (mean ± s.e.m, One-way ANOVA with Bonferroni correction. n = 4,6,5,5 mice each group respectively). (h) BrdU and panCK co-expressing cells can be observed 2 days after ΔMdm2. Representative images are shown are representative of 3 mice each group. Scale bars = 50 µm.
**Supplementary Figure 4** Expandability of EpCAM+ CD24+ CD133+ population in vitro. (a) Morphology of cdHPCs after passaging with trypsin (left) or diluted trypsin (right). Insets show high magnification pictures. (b) Percentage of total EpCAM+CD24+CD133+ cells after in vitro expansion. (mean ± s.e.m, n = 6 biological replicates, Mann-Whitney test) (c) mRNA expression in relative to housekeeping gene (Ppia) of expanded cdHPCs (mean ± s.e.m, n= 3 biological replicates). (d) Heat map representation comparing mRNA expression in relative to housekeeping gene Ppia of cdHPC clones and primary hepatocytes. (e) Relative mRNA expression for HPC related genes Lgr5, EpCAM, Albumin, Ck19, Spp1, Sox9 on early and late passages cdHPCs (mean ± s.e.m, Kruskal Wallis test. P>0.05, except Lgr5 P=0.0286; n=4 biological replicates). (f) Immunocytochemistry for HPC markers Sox9, CK19, OPN, and HNF1β on early and late passages cdHPC. (g) Cell area, roundness, and width to length ratio of early and late passages cdHPCs (mean ± s.e.m, Kruskal Wallis test P=0.0107; n=4 biological replicates). (h) Calculation for the average number of cell division after 10 passages; n=3 biological replicates. Representative images represent data obtained from 3 individual experiments. Scale Bar = 100 µm.
Supplementary Figure 5 Ability to differentiate towards both hepatic and biliary lineage in vitro. (a) FACS analysis of LGR5, CD31 and CD45 expression on in vitro expanded cdHPCs. Isotype control (blue line) (b) Immunocytochemistry for desmin and GFAP in in vitro expanded HPCs. (isolated stellate cells as positive control). (c) In vitro differentiation of expanded HPCs into cholangiocytes stained with activated bile duct marker MIC1C3 (green) and Hnf1β (red), DAPI (blue). (d) In vitro differentiation of expanded HPCs into hepatocytes dotted line demarcates a hepatocyte like colony, upper panel. Increase Glycogen storage detected by Periodic acid-Schiff staining on differentiated cdHPCs. (e) Alb mRNA expression and secreted protein following hepatocyte differentiation (mean ± s.e.m, P = 0.007 Mann-Whitney test; n=5 biological replicates). Lower histograms demonstrate expression of cholangiocyte related genes, and hepatocyte transcription factor Hnf1α. Representative images are shown as representative of 3 individual experiments. Scale Bars = 50 µm.
Supplementary Figure 6 Secondary clone sorting assay for the in vitro expanded HPCs. (a) EpCAM and CD24 expression of in vitro expanded HPCs. (b) EpCAM and CD24 expression analysis of secondary clones 7 days after replating. (c) Percentage of EpCAM+ CD24Hi population in secondary clone cultures (mean ± s.e.m., Kruskal Wallis test; \( P = 0.0076 \)). Data are represented as mean ± s.e.m., n=5 biological replicates. Representative images represents 3 individual experiments.
Supplementary Figure 7  Liver repopulating capacity of the in vitro expanded HPCs. (a) Stitched image of GFP expressing cells in CAG-GFP HPCs transplanted animals. (b) Detection of GFP, ductular marker (panCK) and hepatocyte marker (HNF4α) in transplanted animals and non-transplanted controls (white arrow, GFP- panCK+; red arrow, GFP+ panCK+; green arrow, GFP+ HNF4α--; yellow arrow, GFP+ HNF4α+). (c) Detection of GFP and proliferation marker (Ki67) or senescence marker (p21) in transplanted animals and non-transplanted controls (insets, higher magnification) three months after HPC transplantation (upper panel). (White arrows show GFP+ Ki67+ hepatocytes; Yellow arrow shows p21- GFP+ hepatocytes). Data shown here are representative of 3 experiments with 8-10 mice each group. Scale Bars = 50 µm, except stitched image (a) where scale bar = 200µm.
Supplementary Figure 8 Schematic representation of experimental design for the AhCreΔMdm2 mice.
### Supplementary Table 1
Recombination efficiency of the ΔMdm system and summary of experimental design. Efficiency of recombination as assessed by over-expression of nuclear p53 in hepatocytes; n≥3 each group († by qPCR of genomic DNA from purified ex vivo hepatocytes; see also Supplementary Fig 1). No up-regulation of hepatocellular p53 was seen in uninduced AhCre⁺ Mdm2⁺flox/+ control mice given 80mg/Kg βNF. Mice induced with 240mg/kg βNF were given 3 separate injections of 80mg/Kg βNF over 24hrs. Schematic representation of experimental design for the AhCreΔMdm2 mice.

| MDM2 Genotype | AhCre Genotype | Induction dose (mg/kg) | Recombination efficiency (%)† | Mortality (%) | (days post induction) | Ductular expansion |
|---------------|----------------|------------------------|-------------------------------|--------------|----------------------|------------------|
| flox/flox     | -              | 80                     | 0                             | 0            | N/A                  | -                |
| flox/flox     | +              | 0                      | 0                             | 0            | N/A                  | -                |
| flox/flox     | +              | 20                     | 85.1                          | 17           | 10                   | +                |
| flox/flox     | +              | 40                     | 95.1                          | 83           | 7                    | ++               |
| flox/flox     | +              | 80                     | 98.9 (96.8†)                  | 100          | 8                    | +++              |
| flox/flox     | +              | 240*                   | 99.2                          | 100          | 6                    | +++              |
| flox/-        | +              | 80                     | 98.8                          | 58           | 19                   | +++              |
## List of antibodies used

### Immunohistochemistry

| Antibody | Company | Catalogue Number | Clone | Fixation | Retrieval | Dilution | Secondary Antibody (IgG) |
|----------|---------|------------------|-------|----------|----------|----------|--------------------------|
| EpCAM    | Dako    | Z0622            | EPR15 | Formalin | NaC      | 1/500    | Anti-rabbit              |
| CYP2D6   | Gift from Prof Roland Wolf, University of Dundee, UK | N/A     |       | Formalin | NaC      | 1/500    | Anti-sheep               |
| OPN      | R&D systems | AF808 (polyclonal) |       | Formalin | NaC      | 1/200    | Anti-goat                |
| MIC-1C3  | Novus Biologicals | NBD1-18961 (MIC1-1C3) |       | Formalin | NaC      | 1/200    | Anti-rat                 |
| Fn14     | Abcam   | ab85089          | E11-2  | Formalin | NaC      | 1/200    | Anti-rabbit              |
| Desmin   | Abcam   | ab8592           | E2-11  | Formalin | NaC      | 1/200    | Anti-rabbit              |
| SMA      | Sigma-Aldrich | A2547 (1A4) |       | Formalin | NaC      | 1/1000   | Anti-mouse               |
| GFAP     | Abcam   | ab7260           | E11-2  | Formalin | NaC      | 1/200    | Anti-rabbit              |
| CD24     | Biolegend | 101801 (M1/69) | M1/69 | Formalin | NaC      | 1/200    | Anti-rat                 |

### Flow Cytometry and Fluorescence Activated Cell Sorting (FACS)

| Antibody | Company | Catalogue Number | Clone | Fixation | Retrieval | Dilution | Secondary Antibody (IgG) |
|----------|---------|------------------|-------|----------|----------|----------|--------------------------|
| EpCAM    | EBioscience | 17-5791-80 (G8.8) |       | Formalin | NaC      | 1/200    | N/A                      |
| Dlk      | MBL International | D187-4 (24-11) |       | Formalin | NaC      | 1/100    | N/A                      |
| CD133    | EBioscience | 11-1331-82 (13A4) |       | Formalin | NaC      | 1/50     | N/A                      |
| CD45     | EBioscience | 12-0451-82 (30-F11) |       | Formalin | NaC      | 1/100    | N/A                      |
| CD31     | EBioscience | 12-0311-82 (390) |       | Formalin | NaC      | 1/100    | N/A                      |
| Ter119   | EBioscience | 12-5921-82 (TER-119) |       | Formalin | NaC      | 1/100    | N/A                      |

**Supplementary Table 2** List of antibodies used. Antibodies used for antigen detection in the current study are provided together with mode of tissue fixation, method of antigen retrieval and working dilution. Formalin = fixation for 6 hours in 10% formalin in PBS, NaC = 100mM Sodium Citrate pH 6.0, and Tris EDTA = 100mM Tris EDTA pH 9.0.