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Small molecule drugs promote repopulation of transplanted hepatocytes by stimulating cell dedifferentiation

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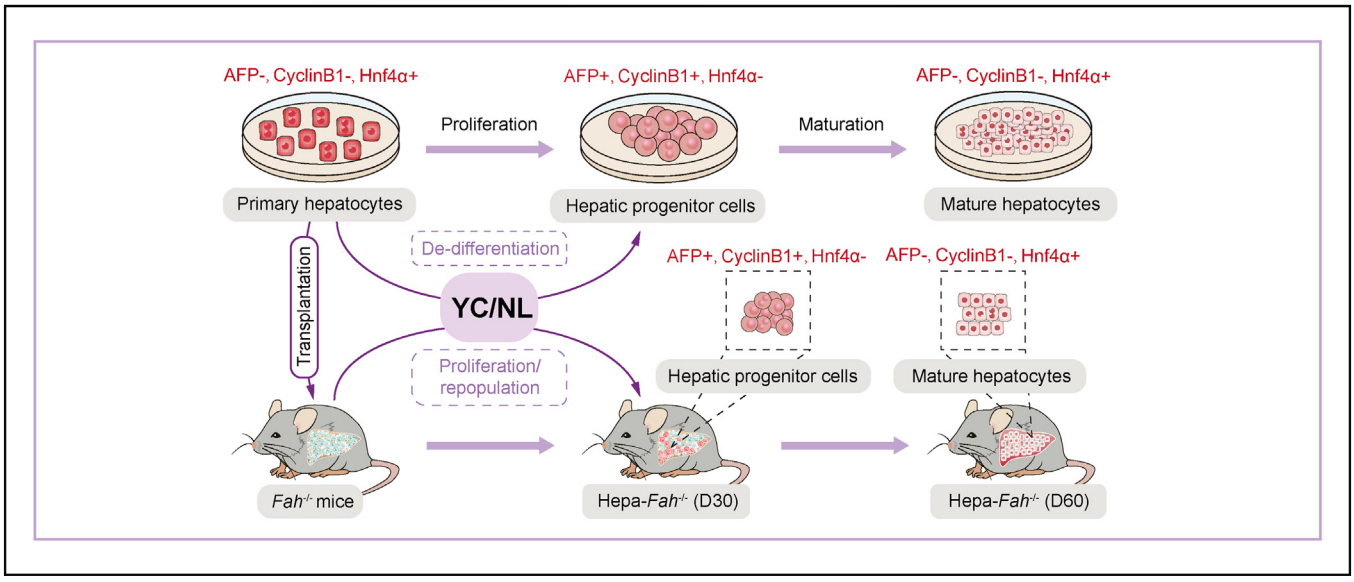
Graphical abstract



Highlights

- Transplanted hepatocytes dedifferentiate into HPCs before repopulation.
- Y-27632 (Y) and CHIR99021 (C) convert mouse hepatocytes into HPCs & support long-term culture (>30 passages) *in vitro*.
- YC stimulate the proliferation of transplanted hepatocytes in *Fah*$^{-/-}$ livers by promoting their conversion into HPCs.
- Two clinically used drugs target the same pathways as YC, also promoting hepatocyte proliferation *in vitro* and *in vivo*.

Impact and implications

Hepatocyte transplantation may be a treatment option for patients with end-stage liver disease. However, one important obstacle to hepatocyte therapy is the low level of engraftment and proliferation of the transplanted hepatocytes. Herein, we show that small molecule compounds which promote hepatocyte proliferation *in vitro* by facilitating dedifferentiation, could promote the growth of transplanted hepatocytes *in vivo* and may facilitate the application of hepatocyte therapy.

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Small molecule drugs promote repopulation of transplanted hepatocytes by stimulating cell dedifferentiation

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Background & Aims: Hepatocyte transplantation has emerged as a possible treatment option for end-stage liver disease. However, an important obstacle to therapeutic success is the low level of engraftment and proliferation of transplanted hepatocytes, which do not survive long enough to exert therapeutic effects. Thus, we aimed to explore the mechanisms of hepatocyte proliferation *in vivo* and find a way to promote the growth of transplanted hepatocytes.
Methods: Hepatocyte transplantation was performed in *Fah*$^{-/-}$ mice to explore the mechanisms of hepatocyte proliferation *in vivo*. Guided by *in vivo* regeneration mechanisms, we identified compounds that promote hepatocyte proliferation *in vitro*. The *in vivo* effects of these compounds on transplanted hepatocytes were then evaluated.
Results: The transplanted mature hepatocytes were found to dedifferentiate into hepatic progenitor cells (HPCs), which proliferate and then convert back to a mature state at the completion of liver repopulation. The combination of two small molecules Y-27632 (Y, ROCK inhibitor) and CHIR99021 (C, Wnt agonist) could convert mouse primary hepatocytes into HPCs, which could be passaged for more than 30 passages *in vitro*. Moreover, YC could stimulate the proliferation of transplanted hepatocytes in *Fah*$^{-/-}$ livers by promoting their conversion into HPCs. Netarsudil (N) and LY2090314 (L), two clinically used drugs which target the same pathways as YC, could also promote hepatocyte proliferation *in vitro* and *in vivo*, by facilitating HPC conversion.
Conclusions: Our work suggests drugs promoting hepatocyte dedifferentiation may facilitate the growth of transplanted hepatocytes *in vivo* and may facilitate the application of hepatocyte therapy.
Impact and implications: Hepatocyte transplantation may be a treatment option for patients with end-stage liver disease. However, one important obstacle to hepatocyte therapy is the low level of engraftment and proliferation of the transplanted hepatocytes. Herein, we show that small molecule compounds which promote hepatocyte proliferation *in vitro* by facilitating dedifferentiation, could promote the growth of transplanted hepatocytes *in vivo* and may facilitate the application of hepatocyte therapy.

Introduction

Globally, liver cancer and cirrhosis are responsible for approximately 2 million deaths each year.[1] For these patients with end-stage liver diseases, liver transplantation is the only treatment option, yet less than 10% of transplantation needs are met by current organ supply.[1] There is a huge gap between suitable donor organs and patients waiting for transplantation.[2] Hepatocyte, instead of whole liver, transplantation is emerging and may overcome the shortages of organs and reduce the need for invasive surgical procedures.[3] However, many obstacles still remain, including limited donor livers, difficulties in isolating good-quality hepatocytes from often suboptimal donor livers and expanding hepatocytes *in vitro*, problems in maintaining hepatocyte viability after cryopreservation, low levels of engraftment and proliferation in transplanted hepatocytes, as well as allograft rejection.[2,3] Studying the mechanism of hepatocyte proliferation and finding ways to promote hepatocyte growth after transplantation may help to remove some of these obstacles.

The liver is normally a quiescent organ. Mature hepatocytes do not cycle and their turnover occurs very slowly over a period of several months.[4] Recent evidence has suggested hepatocytes in all liver zones are able to proliferate in random to maintain homeostasis; thus, a stem/progenitor cell compartment is not

Keywords: Hepatocyte transplantation; Hepatocyte expansion; Dedifferentiation; Hepatocyte progenitor cells; Small molecule compounds.
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required for liver maintenance.[5] However, injury-induced liver regeneration occurs via different mechanisms.[6] Early research demonstrated that hepatic progenitor cells (HPCs, also named oval cells), residing within the Canals of Hering[7] and expressing hepatoblast marker AFP and other biliary genes,[12] serve as an alternative pathway for regeneration during prolonged, severe liver injury when hepatocyte proliferation may be blocked.[8] However, several lineage-tracing studies have shown that pre-existing HPCs contribute little to liver regeneration, virtually all new hepatocytes come from preexisting hepatocytes,[9] and the ability to proliferate is broadly distributed among hepatocytes rather than limited to a rare stem cell-like population.[5] However, these studies did not explore whether hepatocytes dedifferentiate into HPCs before proliferation. Other studies have found that in chronic liver injury, hepatocytes can dedifferentiate into a HPC state to restore the liver mass. In diet-induced chronic liver injury, hepatocytes have been shown to convert to cholangiocyte-like cells ($Sox9^{+}EpCAM^{+}$ cells) and supply new hepatocytes to repair damaged tissues,[10] and around 20% of newly regenerated hepatocytes were derived from hepatocyte-derived HPCs.[11] Another study utilizing hepatocyte-chimeric mice showed that bipotential HPCs were derived from chronically injured mature hepatocytes and could revert back to hepatocytes.[12] Present evidence suggests that HPCs may emerge from hepatocytes via dedifferentiation upon liver injury and contribute to liver regeneration. However, whether such a process also occurs during repopulation after hepatocyte transplantation remains unclear.

Mature hepatocytes were difficult to culture or grow *in vitro*, while cells with HPC characteristics, such as $Lgr5^{+}$ hepatocytes from mice liver[13] or $Epcam^{+}$ hepatocytes from human liver[14] could be expanded *in vitro*. Recently, several studies have shown that small molecule chemicals or cytokines could convert mature hepatocytes into HPCs which can proliferate *in vitro*. In 2017, the combination of small molecules A-83-01 (inhibitor of TGF-β signaling), Y27632 (inhibitor of ROCK kinase) and CHIR99021 (agonist of WNT signaling) were used to grow mice hepatocytes *in vitro* by inducing hepatocyte dedifferentiation.[15] Immediately after, another combination of A-83-01, Y27632, CHIR99021, S1P and LPA were found to induce the conversion of mouse hepatocytes to HPCs and promote growth *in vitro*.[16] One year later, a combination of Wnt3a, A-83-01 and Y27632 was found to support human hepatocyte proliferation via dedifferentiation to HPCs.[17] Then, a study reported that A-83-01 and CHIR99021 were enough to support the dedifferentiation and proliferation of human primary hepatocytes.[18] It seems that inducing hepatocyte dedifferentiation into HPCs is a common mechanism to promote their growth *in vitro*. We wonder whether the conditions used to facilitate hepatocyte to HPC conversion *in vitro* can stimulate hepatocyte growth *in vivo* after transplantation.

Herein, we report that HPC-dependent regeneration occurs in $Fah^{-/-}$ mice receiving hepatocyte transplantation. We also identify that small molecules which promote hepatocyte dedifferentiation *in vitro* can facilitate the growth of transplanted hepatocytes *in vivo*.

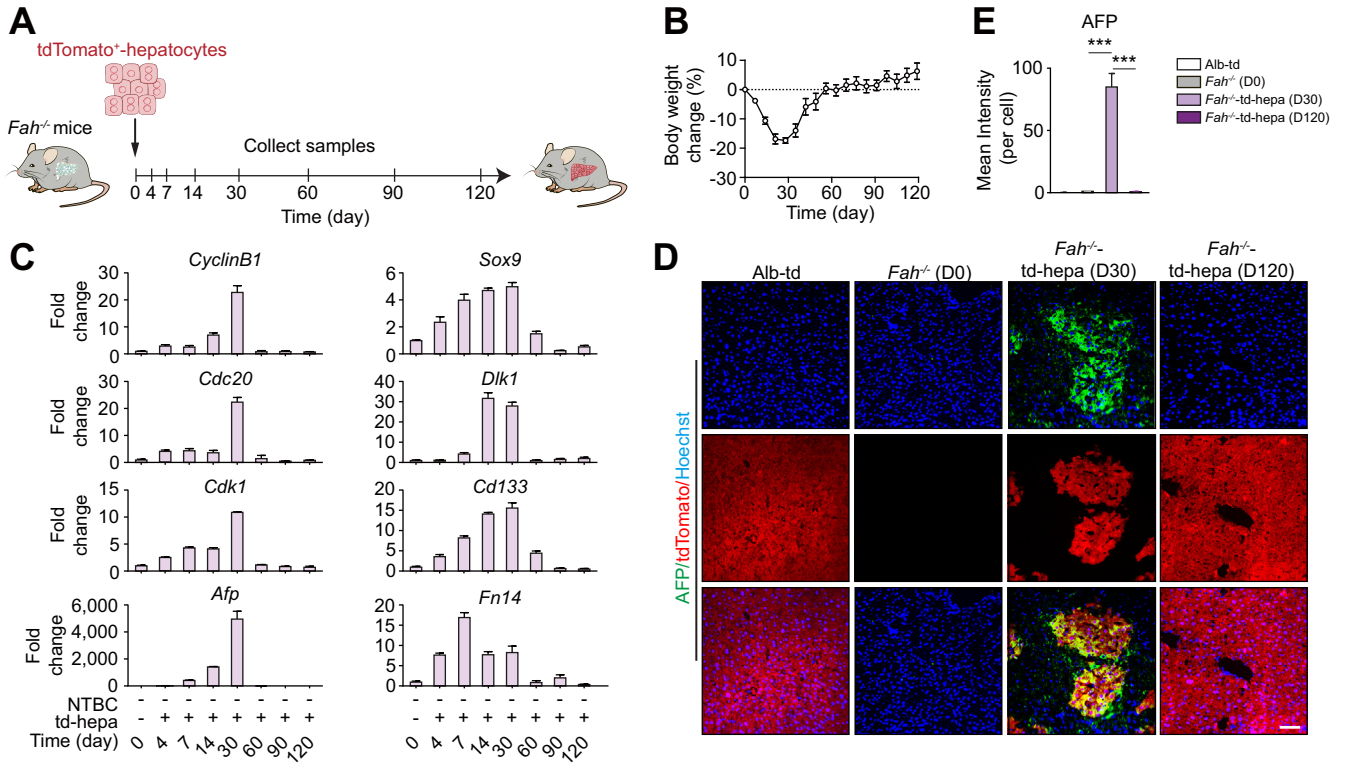

Fig. 1. Hepatocytes were reprogrammed to a progenitor state after transplantation. (A) A schematic view of $Fah^{-/-}$ mice transplanted with td-hepa (B) Body weight change of $Fah^{-/-}$ mice transplanted with td-hepa for 120 days (n = 3) (C) Quantitative RT-PCR analysis of cell cycle genes (*CyclinB1, Cdc20 and Cdk1*) and hepatic progenitor genes (*Afp, Sox9, Dlk1, Cd133 and Fn14*) in liver samples of $Fah^{-/-}$ mice transplanted with td-hepa for 120 days (n = 3) (D) Immunofluorescence staining of AFP in frozen liver sections of $Fah^{-/-}$ mice before transplantation ($Fah^{-/-}$ [D0]), or 30 and 120 days after td-hepa transplantation ($Fah^{-/-}$-td-hepa [D30] and [D120]). The livers of Alb-td mice were used as a control (E) Statistical analysis of AFP staining in (D). All data are means ± SEM. ***p <0.001 (Student's t test).

Materials and methods

Mice

All mice were housed under controlled humidity and temperature conditions and under 12 h light/dark cycles. The care and use of animals complied with international guidelines and were approved by the Animal Ethics Committee of Shanghai Institute of Materia Medica.

Isolation of mice primary hepatocyte

Primary hepatocytes were isolated by the classic two-step collagenase perfusion technique from mice (C57BL/6 J) at the age of 8-10 weeks. R26R^{tdTomato} mice (Jackson Laboratory) were crossed with Albumin-Cre mice and the offspring Albumin-Cre:R26R^{tdTomato} (Alb-td) mice were used for the isolation of tdTomato^{+}-hepatocytes. The liver was perfused through the inferior vena cava with 25 ml perfusion buffer and then 25 ml enzyme buffer. The hepatocytes were then released into the M199 medium (GIBCO) using sterile surgical scissors. Cell suspension was filtered through a 70 μm cell strainer (Corning). After which, hepatocytes were purified with 50% Percoll gradient media (Sigma) at low-speed centrifugation (1,500 rpm, 15 min) then the pellets were dissociated into a single-cell suspension.

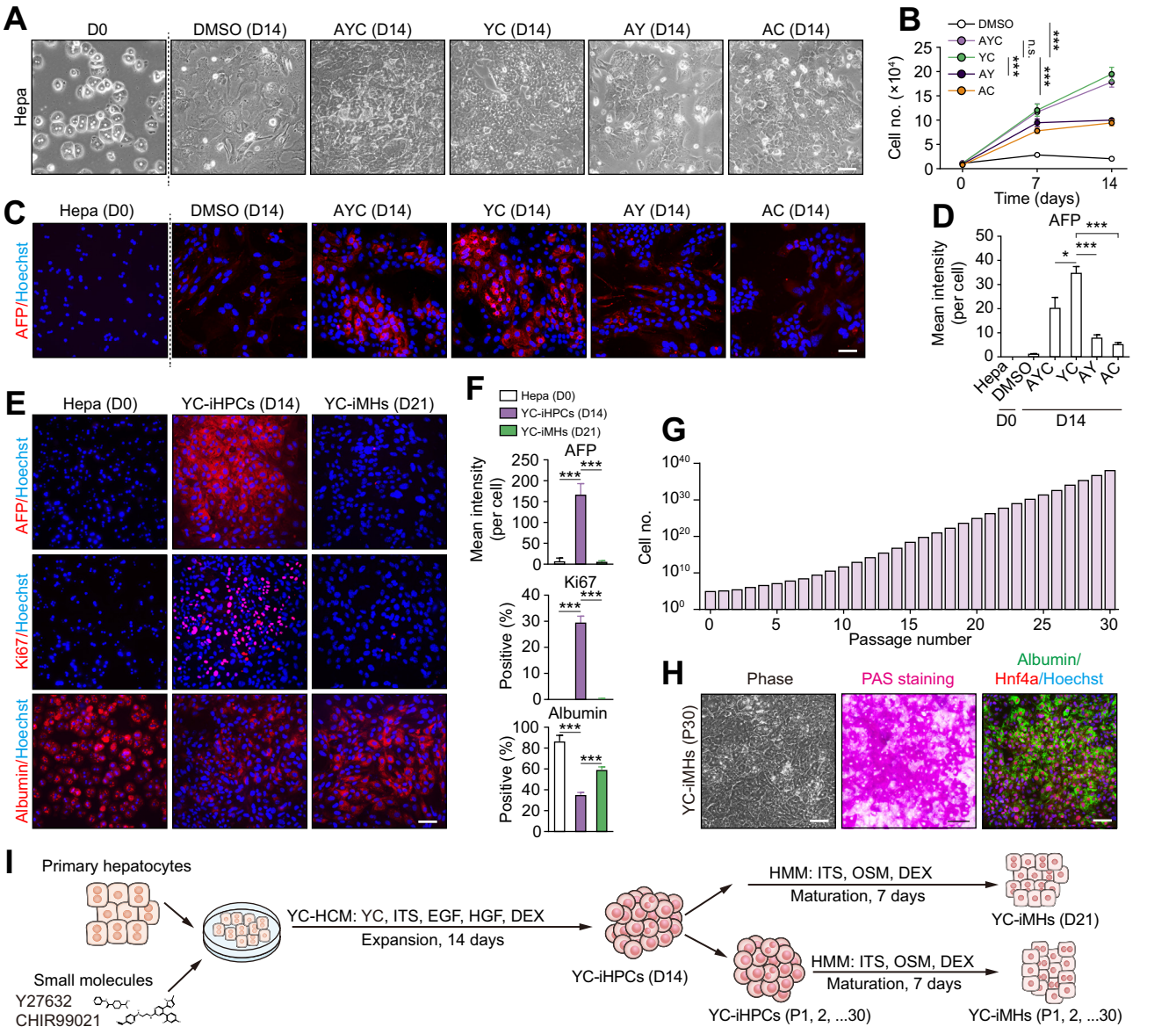

Fig. 2. Reprogramming of hepatocytes into expandable HPCs by Y27632 and CHIR99021. (A, B) Representative morphology (A) and growth curves (B) of primary hepatocytes (D0) cultured in various combinations of A83-01 (A), Y27632 (Y) and CHIR99021 (C) for 14 days (D14) (n = 4). ***p <0.001 (two-way ANOVA) (C) Immunofluorescence staining of AFP in hepatocytes cultured in various combinations of A, Y and C for 14 days (D) Statistical data of the immunofluorescence staining data in (C) (eight random fields for each group) (E) Immunofluorescence staining of AFP, Ki67 and albumin in Hepa (D0), YC-iHPCs (D14) and YC-iMHs (D21) (F) Statistical data of AFP, Ki67 and albumin in (E) (eight random fields for each group) (G) Calculated cumulative cell numbers of YC-iHPCs for 30 passages (H) Representative images of morphology, PAS staining, and immunofluorescence staining of albumin and Hnf4α in YC-iMHs (P30) (I) A schematic view of hepatocyte expansion using chemical cocktail YC. Nuclei were stained with Hoechst 33342. Scale bars represent 100 μm. All data are means ± SEM. *p <0.05, **p <0.01, ***p <0.001 (Student's t test unless specified otherwise).

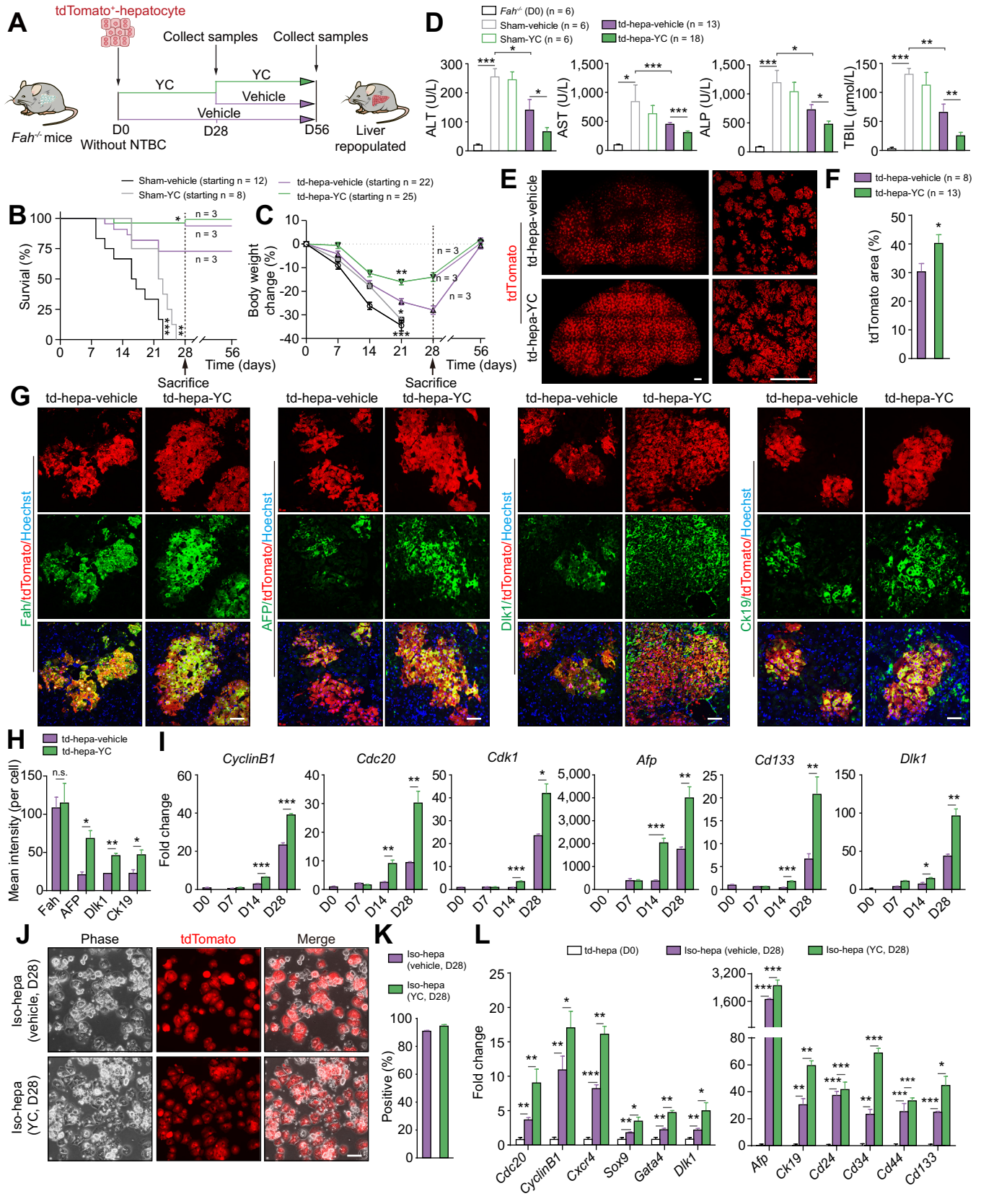

Fig. 3. Y27632 and CHIR99021 promote proliferation of transplanted hepatocytes *in vivo*. (A) A schematic view of YC treatment in *Fah*$^{-/-}$ mice transplanted with td-hepa (B, C) Survival curves (B) and body weight change (C) of *Fah*$^{-/-}$ mice receiving vehicle (sham-vehicle, starting n = 12) or YC (sham-YC, starting n = 8), or *Fah*$^{-/-}$ mice transplanted with td-hepa and then treated with vehicle (td-hepa-vehicle, starting n = 22) or YC (td-hepa-YC, starting n = 25) for 56 days after NTBC withdrawal. Most of the surviving mice were sacrificed for analysis on D28. Three of the surviving mice in the vehicle group continued to receive vehicle until

The viability of isolated hepatocytes was about 90% as determined by Trypan blue staining. For further details please see the supplementary materials and methods.

Hepatocyte transplantation and sample collection

Fah$^{-/-}$ mice were fed with 7.5 mg/L 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione (NTBC) in drinking water, *Fah*$^{-/-}$ mice at the age of 8-12 weeks were used for transplantation. For transplantation, hepatocytes (2.5×10^6) were suspended in 200 μl of 0.9% sodium chloride solution and transplanted into *Fah*$^{-/-}$ mice via intrasplenic injection through a left-flank incision under 1.25% tribromoethanol anesthesia. After the operation, NTBC was withdrawn from the drinking water. After transplantation, *Fah*$^{-/-}$ mice were treated with vehicle (0.5% sodium carboxymethyl cellulose in PBS), YC (10 mg/kg Y27632 and 14 mg/kg CHIR99021, per os every day) or NL (0.3 mg/kg Netarsudil and 3 mg/kg LY2090314, per os every day). The blood and liver samples were collected at indicated time points. Total serum bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) were measured (Bioassay system kit).

Statistical analysis

Values are reported as the means ± SEM. *p* values were calculated with Student's *t* test, two-way ANOVA test or log-rank test as indicated in the figure legends, $p < 0.05$ was considered statistically significant. All graphs were plotted with GraphPad Prism software. The Immunofluorescence images were analyzed using ImageJ software.

Results

Hepatocytes are reprogrammed to a hepatic progenitor state during repopulation *in vivo*

To trace hepatocytes after transplantation *in vivo*, tdTomato$^+$-hepatocytes (td-hepa) were isolated from mice obtained by crossing R26R^{tdTomato} mice with the *Albumin*-Cre mice[19] (Fig. S1A,B). *Fah*$^{-/-}$ mice require NTBC for survival.[20] After NTBC withdrawal, *Fah*$^{-/-}$ mice experience liver failure and die in about 30 days unless they receive hepatocyte transplantation.[21,22] This is an ideal model to study the proliferation and function of transplanted hepatocytes *in vivo*.[12] Therefore, after NTBC withdrawal, td-hepa were transplanted into *Fah*$^{-/-}$ mice via intrasplenic injection through a left-flank incision under tribromoethanol anesthesia (Fig. 1A). The body weight of these animals kept dropping in the first 3 weeks, before gradually recovering (Fig. 1B). Liver samples of *Fah*$^{-/-}$ mice receiving td-hepa were collected at different time points (day [D] 4, 7, 14, 30, 60, 90 and 120). Compared to the livers of *Fah*$^{-/-}$ mice before NTBC withdrawal, the cell cycle genes (*CyclinB1, Cdc20* and *Cdk1*) and HPC genes (*Afp, Sox9, Dlk1, Cd133* and *Fn14*) in the livers of *Fah*$^{-/-}$ mice receiving td-hepa were upregulated from D4, reached a peak at around D30 and then decreased to normal (Fig. 1C). The td-hepa showed clonal expansion at D30 and almost fully occupied the liver at D120 (Fig. 1D and Fig. S1C). It is not surprising that only the td-hepa were positive for Fah staining (Fig. S1C and D). Interestingly, only the repopulating (D30) td-hepa in *Fah*$^{-/-}$ mice were positive for AFP staining but not the repopulated (D120) td-hepa (Fig. 1D,E). These results suggest that the transplanted hepatocytes dedifferentiate to an HPC stage to proliferate and then redifferentiate after repopulation.

Reprogramming of hepatocytes into expandable HPCs by Y27632 and CHIR99021

A previous study has shown that a cocktail of small molecules A-83-01 (inhibitor of TGF-β signaling), Y27632 (inhibitor of ROCK kinase) and CHIR99021 (agonist of Wnt signaling) (AYC) can convert rat and mouse hepatocytes into HPCs with high proliferative capacity.[15] We wondered whether the combination could be simplified to facilitate *in vivo* application. To optimize the chemicals, we cultured hepatocytes in the classical hepatocyte culture medium (containing epidermal growth factor and hepatocyte growth factor) supplemented with AYC or any two of the three chemicals (YC, AY and AC). Consistent with current knowledge,[15,23] no proliferation of hepatocytes was observed in the vehicle (DMSO)-treated group at D14 (Fig. 2A,B). As expected, AYC stimulated significant proliferation as the cell number increased about 20 times, which was similar to a previous report[15] (Fig. 2A,B). Interestingly, YC was found to induce a similar speed of hepatocyte growth as AYC, while AY or AC could only promote moderate proliferation (Fig. 2A,B). The proliferating cells in YC and AYC groups at D14 show typical epithelial morphology with a high nucleus/cytoplasm ratio, which is a typical feature of HPCs[15] (Fig. 2A). Immunofluorescence staining revealed that the proliferating cells in the YC group expressed the highest level of AFP and Dlk1, which was also expressed in the AYC group, but absent in fresh isolated hepatocytes, or cells cultured for 14 days in other conditions (Fig. 2C,D, Fig. S2A,B). Quantitative reverse-transcription (RT-PCR) analysis also confirmed more cell cycle genes and HPC genes were upregulated in YC-induced HPCs (YC-iHPCs) (D14) (Fig. S2C and D). Compared to hepatocytes, the proliferating cells in the YC group were also highly positive for the cell cycle marker Ki67 but showed significantly reduced expression of the mature hepatocyte marker albumin (Fig. 2E,F). Hence, these cells were named YC-iHPCs. Moreover, YC-iHPCs (D14) could be

D56. Three of the mice in the YC group continued to receive YC until D56, and another three in the YC group started to receive vehicle from D28-D56. Statistical differences between the two groups were analyzed from D0-D21. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. td-hepa-vehicle group (log-rank test for B and two-way ANOVA test for C) (D) Serum levels of ALT, AST, ALP and TBIL in the *Fah*$^{-/-}$ mice after transplantation, animal numbers were the same as the survived mice in (C); *Fah*$^{-/-}$ mice before NTBC withdrawal (*Fah*$^{-/-}$ (D0)) were used as controls. (E) Representative tdTomato images of the whole liver (left) and frozen sections of liver (right) of td-hepa-vehicle and td-hepa-YC mice (D28), scale bar represents 1 mm (F) Quantitative analysis of tdTomato-positive areas in (E) (td-hepa-vehicle group, n = 8, td-hepa-YC group, n = 13) (G) Immunofluorescence staining of Fah and progenitor markers (AFP, Dlk1 and Ck19) in frozen liver sections of td-hepa-vehicle and td-hepa-YC mice (D28) (H) Statistical analysis of the intensity of Fah, AFP, Dlk1 and Ck19 staining in (G) (n = 3) (I) Quantitative RT-PCR analysis of cell cycle genes (*CyclinB1, Cdc20, Cdk1*) and hepatic progenitor genes (*Afp, Cd133 and Dlk1*) in livers of td-hepa transplanted *Fah*$^{-/-}$ mice treated with vehicle or YC at D7, D14 and D28 (n = 3). *Fah*$^{-/-}$ before NTBC withdrawal (D0) was used as control (J) Representative morphology and fluorescence images of td-hepa isolated (by FACS) from td-hepa-vehicle and td-hepa-YC mice (D28) (K) Statistical analysis of tdTomato$^+$ hepatocytes in (J) (n = 3) (L) Quantitative RT-PCR analysis of cell cycle genes (*Cdc20* and *CyclinB1*) and hepatic progenitor genes (*Cxcr4, Sox9, Gata4, Dlk1, Afp, Ck19, Cd24, Cd34, Cd44,* and *Cd133*) in td-hepa isolated from td-hepa-vehicle and td-hepa-YC mice (D28) (n = 3). Primary td-hepatocytes were used as controls. Nuclei were stained with Hoechst 33342. Scale bars represent 100 μm. All data are means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t* test unless specified otherwise).

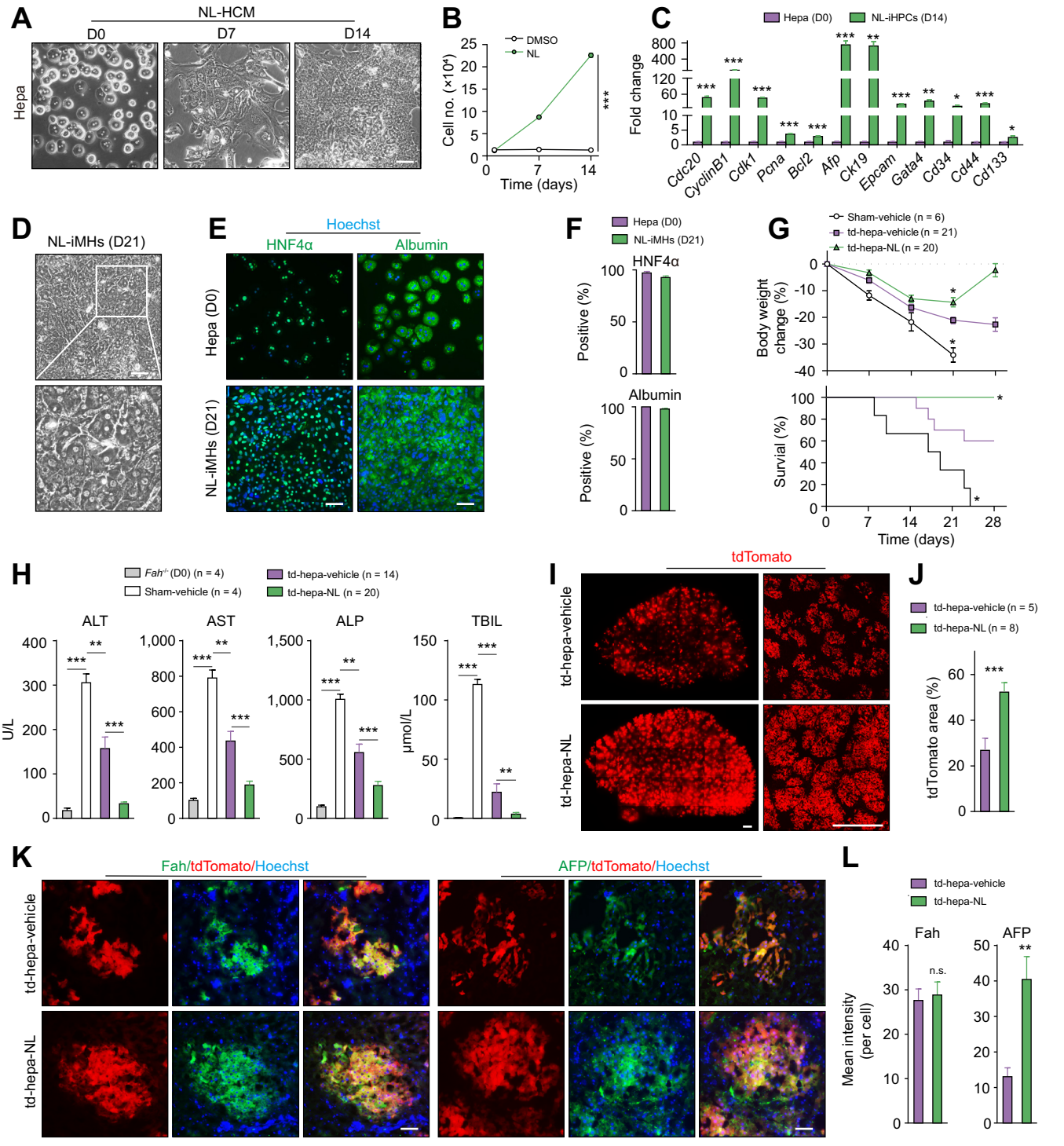

Fig. 4. Netarsudil and LY2090314 promote hepatocyte expansion *in vitro* and repopulation *in vivo*. (A, B) Representative images (A) and growth curves (B) of primary hepatocytes cultured with Netarsudil (N, 0.1 μM) and LY2090314 (L, 1 μM) for 0-14 days (n = 3), ***p <0.001 (two-way ANOVA) (C) Quantitative RT-PCR analysis of cell cycle genes (*Cdc20, CyclinB1, Cdk1, Pcna,* and *Bcl2*) and hepatic progenitor genes (*Afp, Ck19, Epcam, Gata4, Cd34, Cd44 and Cd133*) in NL-iHPCs (D14) (n = 3). Primary td-hepatocytes were used as controls (D) Representative phase contrast images of NL-iMHs (D21) (E, F) Immunofluorescence staining (E) and statistical analysis (F) of HNF4α and albumin in Hepa (D0) and NL-iMHs (D21) (eight random fields for each group) (G) Body weight change and survival curves of *Fah*$^{-/-}$ mice without transplantation (n = 6), or receiving td-hepa transplantation and then treated with vehicle (td-hepa-vehicle, n = 21) or NL (td-hepa-NL, n = 20) for 28 days after NTBC withdrawal. Statistical differences between two groups were analyzed from D0-D21 for body weight change, *p <0.05 *vs.* td-hepa-vehicle group (two-way ANOVA for body weight change, log-rank test for survival curves) (H) Serum levels of ALT, AST, ALP and TBIL in the *Fah*$^{-/-}$ mice without transplantation (sham-vehicle, n = 4), or *Fah*$^{-/-}$ mice transplanted with td-hepa and then treated with vehicle (td-hepa-vehicle, n = 14) or NL (td-hepa-NL, n = 20). *Fah*$^{-/-}$ mice before NTBC withdrawal (*Fah*$^{-/-}$ (D0), n = 4) were used as controls (I) Representative tdTomato images of the whole liver (left) and frozen sections of liver (right) of td-hepa-transplanted *Fah*$^{-/-}$ mice receiving vehicle or NL (D28), scale bar represents 1 mm (J) Quantitative analysis of tdTomato-positive areas in (I)

differentiated into mature hepatocytes with a widely used hepatic maturation medium (HMM)[15] with minor modifications. After culturing YC-iHPCs (D14) in HMM for 7 days, YC-iHPCs transformed into cells with typical mature hepatocyte morphology (Fig. S2E and F), which were named YC-induced mature hepatocytes (YC-iMHs). The YC-iMHs were negative for AFP and Ki67, but albumin was significantly increased (Fig. 2E,F). Quantitative RT-PCR analysis also confirmed more HPC genes and cell cycle genes were upregulated in YC-iHPCs (D14) but greatly reduced in YC-iMHs (D21) (Fig. S2G), while the genes related to mature hepatocyte functions were downregulated in YC-iHPCs (D14) but then upregulated in YC-iMHs (D21) (Fig. S2H). To test whether YC could support long-term culture, YC-iHPCs (D14) were passaged every 5 to 7 days (~90% confluence) in YC-supplemented hepatocyte culture medium. YC-iHPCs could be passaged more than 30 times and the cumulative cell number increased from 1×10^5 to about 1×10^{40} in ~150 days without any apparent morphological changes (Fig. 2G and Fig. S2I). YC-iHPCs at passage 10 and 30 expressed high levels of the HPC markers Sox9 and Ck19 and cell cycle markers CyclinD1 and Ki67 (Fig. S2J,K). Importantly, YC-iHPCs at passage 30 could be differentiated into YC-iMHs which were highly positive for Periodic Acid-Schiff staining and expressed high levels of albumin and HNF4α (Fig. 2H). The strategy of using YC to promote hepatocyte proliferation *in vitro* was summarized in Fig. 2I. Taken together, the combination of two chemicals (YC) is enough to reprogram mature hepatocytes into expandable HPCs *in vitro*, and these cells could be redifferentiated into more mature hepatocyte-like cells.

Combination of Y27632 and CHIR99021 promotes hepatocyte proliferation *in vivo*

Next, we sought to evaluate whether YC could promote hepatocyte proliferation *in vivo*. According to the concentration of Y27632 (10 μM) and CHIR99021 (3 μM) used in vitro, Y27632 (10 mg/kg) and CHIR99021 (14 mg/kg) were tested in *Fah*$^{-/-}$ mice by oral administration (Fig. 3A). After NTBC withdrawal, all *Fah*$^{-/-}$ mice without transplantation, who were given vehicle (Sham-vehicle) or YC (Sham-YC), showed continuous body weight loss (Fig. 3C) and died in about 25 days (Fig 3B). In contrast, *Fah*$^{-/-}$ mice receiving td-hepa and then treated with vehicle (td-hepa-vehicle) showed significantly less body weight loss (Fig. 3C), and 16 out of 22 *Fah*$^{-/-}$ mice survived for more than 1 month (Fig. 3B). *Fah*$^{-/-}$ mice receiving td-hepa and YC treatment (td-hepa-YC) showed even less body weight loss (Fig. 3C), and 24 out of 25 *Fah*$^{-/-}$ mice survived for more than 1 month, the survival rate was significantly improved comparing to td-hepa-vehicle group (Fig. 3B). The surviving animals were sacrificed for analysis on D28 or D56 (Fig. 3A,B). The highly increased serum levels of AST, ALT, ALP and total bilirubin due to NTBC withdrawal were significantly reduced in the td-hepa-vehicle group (Fig. 3D) and were further reduced in the td-hepa-YC group (Fig. 3D) at D28. The repopulated td-hepa in YC-treated mice were significantly more abundant than in the vehicle group (Fig. 3E and 3F) at D28. The repopulated td-hepa at D28 were almost 100% positive for Fah staining in both groups (Fig. 3G,H) at D28. However, the td-hepa in YC-treated animals expressed higher levels of the HPC markers AFP, Dlk1 and Ck19 than that in vehicle-treated mice (Fig. 3G,H) at D28. Quantitative RT-PCR confirmed that the expression of cell cycle genes (*CyclinB1, Cdc20* and *Cdk1*) and HPC genes (*Afp, Cd133* and *Dlk1*) were gradually increased in livers after transplantation (Fig. 3I), and YC treatment further upregulated these genes (Fig. 3I). The repopulated td-hepa were isolated by FACS at D28 after transplantation, more than 90% of the cells were tdTomato positive (Fig. 3J and 3K). Quantitative RT-PCR confirmed that cell cycle genes (*Cdc20, CyclinB1*) and HPC genes (*Cxcr4, Sox9, Gata4, Dlk1, Afp, Ck19, Cd24, Cd34, Cd44* and *Cd133*) were significantly upregulated in the YC-treated group (Fig. 3L).

To further confirm that YC stimulated growth of transplanted hepatocytes via dedifferentiation, the global gene expression profiles in the livers were compared. Gene set-enrichment analysis showed there was a clear enrichment of genes related to cell cycle regulation, stem cell proliferation and somatic stem cell population maintenance in the td-hepa-YC group compared to the td-hepa-vehicle group at D28 (Fig. S3A). Transcriptomic comparison was also carried out among *Fah*$^{-/-}$ (D0, before NTBC removal), td-hepa-vehicle and td-hepa-YC groups at D28 (Fig. S3B). Genes related to cell cycle regulation, stem cell proliferation and somatic stem cell population maintenance were upregulated in livers receiving td-hepa transplantation and were even higher in the YC-treated group (Fig. S3B).

Combination of Y27632 and CHIR99021 does not affect hepatocyte engraftment and maturation

At D56, the surviving transplanted mice had been treated with vehicle or YC for 56 days, or treated with YC for 28 days and then treated with vehicle for another 28 days (Fig. 3A). The livers from all three groups at D56 were as normal as the Alb-td-mice (Fig. S4A), td-hepa repopulated more than 95% of the liver in these animals (Fig. S4A). The highly increased serum levels of AST, ALT, ALP and total bilirubin after NTBC withdrawal were almost returned to normal after transplantation (Fig. S4B). The repopulated td-hepa in *Fah*$^{-/-}$ mice were almost 100% positive for Fah, Albumin and Cyp1a2 staining (Fig. S5A-C). The repopulated td-hepa expressed pericentral marker Cyp2e1 only in the pericentral region (Fig. S5D) , in line with previous studies.[24] At D56, the transplanted td-hepa in *Fah*$^{-/-}$ mice were negative for HPC markers AFP and Dlk1 staining (Fig. S5E and F).

Successful hepatocyte transplantation requires efficient engraftment (typically within 3 days after transplantation) and then repopulation.[25] A previous study[25] has demonstrated that blocking macrophage-mediated elimination of transplanted hepatocytes facilitates engraftment and later-on repopulation. We also assessed whether YC treatment affects hepatocyte engraftment. *Fah*$^{-/-}$ mice receiving td-hepa were treated with YC or vehicle for 3 days and the engraftment of td-hepa did not show a significant difference between YC and vehicle groups (Fig. S6A and S6B). Taken together, these results indicate that YC treatment does not affect the engraftment stage and the

(td-hepa-vehicle group, n = 5, td-hepa-NL group, n = 8) (K) Immunofluorescence staining of Fah and AFP in frozen liver sections of td-hepa-transplanted *Fah*$^{-/-}$ mice receiving vehicle or NL (D28) (L) Statistical analysis of the intensity of Fah and AFP staining in (K). Nuclei were stained with Hoechst 33342. Scale bars represent 100 μm. All data are means ± SEM. $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 (Student's *t* test unless specified otherwise).

maturation after repopulation, but only affects the proliferation stage.

Netarsudil and LY2090314 promote hepatocyte proliferation *in vitro* and repopulation *in vivo*

Y27632 and CHIR99021 have not been tested in clinical studies. For possible future clinical application, we aimed to identify an effective combination of available drugs. Netarsudil (N) is also a ROCK inhibitor which is used to treat open-angle glaucoma in the clinic,[26] and LY2090314 (L) is a WNT agonist which has been tested in phase II clinical trials to treat leukemia.[27] So, we tested the combination of different concentrations of N (0 to 0.1 μM) and L (0 to 3 μM) in hepatocyte culture (Fig. S7A), and the combination of N at 0.1 μM and L at 1 μM yielded the best result (Fig. S7B and C), so this NL combination was used for further studies. The primary hepatocytes cultured in NL grow rapidly (Fig. 4A,B). The proliferating cells at D14 showed similar morphology as YC-iHPCs and expressed high levels of cell cycle genes (*Cdc20, CyclinB1, Cdk1, Pcna and Bcl2*) and HPC genes (*Afp, Ck19, Epcam, Gata4, Cd34, Cd44, Cd133*), so these cells were named NL-iHPCs (Fig. 4C). After culturing in HMM for 7 days, the NL-iHPCs could be differentiated into mature hepatocytes (NL-iMHs, Fig. 4D). Immunofluorescence staining revealed that NL-iMHs were highly positive for HNF4α and albumin at levels comparable to primary hepatocytes (Fig. 4E,F). Corresponding doses of N (0.3 mg/kg) and L (3 mg/kg) were then tested in *Fah*$^{-/-}$ mice by oral administration in the same way as YC (Fig. 3A), but with all mice sacrificed at D28. Similar to YC treatment, *Fah*$^{-/-}$ mice receiving td-hepa and then treated with NL (td-hepa-NL) showed significantly less body weight loss and no animal death compared to the td-hepa-vehicle group, which already showed significant therapeutic effect compared to the sham-vehicle group (Fig. 4G). Serum levels of AST, ALT, ALP and total bilirubin were further reduced by the treatment of NL in mice receiving td-hepa transplantation (Fig. 4H), and the repopulated td-hepa in NL-treated mice were significantly more abundant than in the vehicle-treated group (Fig. 4I,J). Immunofluorescence staining revealed that the repopulated td-hepa at D28 were almost 100% positive for Fah staining, and the levels were similar in both NL- and vehicle-treated groups (Fig. 4K,L), but the AFP level was significantly higher in repopulated td-hepa in the NL-treated animals (Fig. 4K,L). Taken together, these results indicate that the drug combination NL could enhance the repopulation of hepatocytes *in vivo* by promoting the reprogramming of hepatocytes into HPCs.

Discussion

Herein, we demonstrate that transplanted hepatocytes can undergo dedifferentiation to HPCs and then convert back to a mature state after repopulation. Small molecules that can induce hepatocyte to HPC conversion *in vitro* can be used to stimulate the same process *in vivo* after hepatocyte transplantation, facilitating growth and repopulation. It is also possible that the same strategy can be used to stimulate *in situ* liver regeneration in various types of liver injury if hepatocyte dedifferentiation is the major route of regeneration.

However, whether the regeneration of the liver after various types of injury requires hepatocyte-HPC-hepatocyte conversion remains to be debated. In partial hepatectomy (PHx), a hepatocyte fate-tracing study has shown that about 98% of newly formed hepatocytes were derived from preexisting hepatocytes, and that the remaining small fraction might be derived from preexisting HPCs.[28] A recent study using single-cell RNA-sequencing and ATAC-sequencing showed that after PHx, some hepatocytes acquired chromatin landscapes and transcriptomes similar to fetal hepatocytes, suggesting dedifferentiation to HPCs.[29] Liver regeneration in models of carbon tetrachloride-induced acute or chronic injury has been shown to be attributable to the proliferation of mature hepatocytes.[30] In another toxin-induced liver injury study, genetic labeling suggested that hepatocytes were the source for hepatocyte renewal and regeneration,[9] although whether these hepatocytes pass through a HPC stage remains unclear. Another study, based on a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet-induced model of liver injury, indicated that mature hepatocytes may convert into cholangiocyte-like cells, which serve as hepatic progenitors for clonal proliferation and can then differentiate into functional hepatocytes.[10] Another study also reported that bipotential adult liver progenitors are derived from chronically injured mature hepatocytes.[12] It seems hepatocyte to HPC conversion is a rather common phenomenon after liver injury and may facilitate regeneration. In our transplantation model, it was very clear that HPC markers gradually increased and peaked at D30 and, at that time point, most of the transplanted cells expressed AFP, indicating the conversion of hepatocytes to HPCs, accompanying cell growth. So, promoting hepatocyte dedifferentiation may also facilitate the regeneration of the liver following other injuries.

A number of pathways have been proposed to drive hepatocyte to HPC dedifferentiation *in vivo*, including Notch, WNT and YAP signaling pathways. One study using mice with a liver-specific deletion of *RBP-Jκ* (an essential component of the canonical Notch pathway) has demonstrated that Notch signaling is required for hepatocyte reprogramming under DDC diet-induced liver injury.[31] *Hes1*, a target gene of *RBP-Jκ*, is also important for the conversion of hepatocytes into primitive ductular cells in DDC-treated chronically injured livers.[32] Conditional knockout of *Ctnnb1* has been shown to reduce the number of HPCs in DDC diet-induced liver injury, demonstrating that the WNT/β-catenin pathway plays a key role in the proliferation of HPCs.[33] Similarly, *Ctnnb1* overexpression results in a higher number of proliferating HPCs in DDC diet-induced liver injury.[34] The YAP-driven transcriptional program has also been reported to be crucial for the process of liver regeneration after DDC injury and specifically for the reprogramming of hepatocytes towards a progenitor, biliary-like fate.[35] Overexpression of active YAP in hepatocytes may drive reprogramming via Notch2 transcriptional regulation, suggesting that YAP-Notch is indeed a crucial axis for this process.[36]

Many of the compounds/supplements used to induce *in vitro* hepatocyte to HPC conversion also target these pathways. In our study, CHIR99021 and LY2090314 are inhibitors of GSK-3β, which may lead to WNT pathway activation. Activation of WNT/β-catenin signaling has also been reported to promote hepatocyte proliferation and liver regeneration in PHx, by upregulating cell cycle regulators.[37] Y27632 and Netarsudil are ROCK inhibitors. ROCK regulates cellular growth, adhesion, migration, metabolism, and apoptosis through control of actin cytoskeletal assembly and cell contraction.[38] There are limited reports on the direct role of the ROCK pathway in inducing hepatocyte to HPC dedifferentiation. However, ROCK inhibitors have now been recognized as useful tools to promote the survival of multiple types of stem cells.[39]

Up to now, we have observed *Fah*$^{-/-}$ mice receiving td-hepa for 170 days (the first 56 days receiving YC treatment) and found that the livers of these mice are normal. But we believe the safety of such treatment should be assessed in large-scale and long-term experiments in the future. In conclusion, drugs/conditions promoting hepatocyte dedifferentiation may promote the growth of transplanted hepatocytes *in vivo* and may facilitate the application of hepatocyte therapy.

Abbreviations

A, A-83-01; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, CHIR99021; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; HMM, hepatic maturation medium; (i)HPCs, (induced) hepatic progenitor cells; iMHs, induced mature hepatocytes; L, LY2090314; N, netarsudil; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclo-hexanedione; PHx, partial hepatectomy; RT-PCR, reverse-transcription PCR; Y, Y27632.

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Conflict of interest

The authors declare no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

J.M. and R.G. conducted most of the experiments, analyzed the results, and wrote the paper; Y.A. G.W. P.T. X.J. B. H. and Q.Y. provided technical assistance with animal studies; X.X. conceived the idea for the project, supervise the study, analyzed the results and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Data availability statement

All data files are available upon request.

Supplementary data

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