Genetic tracing of hepatocytes in liver homeostasis, injury, and regeneration

Yue Wang‡§, Xiuzhen Huang‡§, Lingjuan He‡§, Wenjuan Pu‡§, Yan Li‡§, Qiaozhen Liu‡§, Yi Li‡§, Libo Zhang‡§, Wei Yu‡§, Huan Zhao‡§, Yinggun Zhou‡, and Bin Zhou‡§**

From 1The State Key Laboratory of Cell Biology, CAS Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China, the 2Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China, the 3Department of Gastroenterology, Shanghai 10th People’s Hospital, Tongji University School of Medicine, Shanghai 200072, China, the 4Key Laboratory of Regenerative Medicine of Ministry of Education, Institute of Aging and Regenerative Medicine, Jinan University, Guangzhou 510632, China, and the 5School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China

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The liver possesses a remarkable capacity to regenerate after damage. There is a heated debate on the origin of new hepatocytes after injuries in adult liver. Hepatic stem/progenitor cells have been proposed to produce functional hepatocytes after injury. Recent studies have argued against this model and suggested that pre-existing hepatocytes, rather than stem cells, contribute new hepatocytes. This hepatocyte-to-hepatocyte model is mainly based on labeling of hepatocytes with Cre-recombinase delivered by the adeno-associated virus. However, the impact of virus infection on cell fate determination, consistency of infection efficiency, and duration of Cre-virus in hepatocytes remain confounding factors that interfere with the data interpretation. Here, we generated a new genetic tool Alb-DreER to label almost all hepatocytes (>99.5%) and track their contribution to different cell lineages in the liver. By “pulse-and-chase” strategy, we found that pre-existing hepatocytes labeled by Alb-DreER contribute to almost all hepatocytes during normal homeostasis and after liver injury. Virtually all hepatocytes in the injured liver are descendants of pre-existing hepatocytes through self-expansion. We concluded that stem cell differentiation is unlikely to be responsible for the generation of a substantial number of new hepatocytes in adult liver. Our study also provides a new mouse tool for more precise in vivo genetic study of hepatocytes in the field.

Adult mammalian tissues rely on diverse mechanisms to maintain function and mass. It is well established that organs can maintain homeostasis via either cellular replication or differentiation from stem cells. The liver has a remarkable capacity for regeneration (1). It has been reported that liver regeneration can be driven not only by hepatocytes but also by facultative stem cells under certain injury conditions (2). During chronic and acute injuries, differentiated hepatocytes re-enter the cell cycle, proliferate, and replenish the lost tissue. Based on in vitro and in vivo experiments, bipotential hepatobiliary progenitors (often called oval cells) were proposed as the main source of new hepatocytes and ductal cells under conditions that interfere with hepatocyte proliferation (3–6). Leclercq et al. (3) performed lineage tracing experiments using OPN-iCreERT2;Rosa26RFP mice to show that liver progenitor cells or biliary cells terminally differentiate into functional hepatocytes in mice with liver injury. Clevers and co-workers (4) used Lgr5-IREs-CreERT2;Rosa26-lacZ mice to find that Lgr5-lacZ is not expressed in healthy adult liver; however, small Lgr5-LacZ+ cells appear near bile ducts upon damage, coinciding with robust activation of Wnt signaling. By lineage tracing, they demonstrated that these Lgr5-LacZ+ cells generate hepatocytes and biliary duct cells during the repair phase, indicating that Lgr5+ cells as bipotent liver progenitors (4). Recent studies showed that Sox9+ biphenotypic hepatocytes were derived from mature hepatocytes, and some of them were incorporated into ductular structures, whereas they efficiently differentiate to functional hepatocytes (5). Therefore, biphenotypic hepatocytes not only terminally convert to cholangiocytes but also differentiate back to mature hepatocytes. Mature epithelial cells can show plasticity upon severe injuries and contribute to regeneration (5). In addition, activation of Notch is sufficient to reprogram hepatocytes into biliary epithelial cells (6).

In contrast to these findings, recent studies using genetic lineage-tracing experiments suggested that oval cells contribute minimally to hepato-
to lineage trace Hnf1

Sox9

Their data showed that bipotential hepatic progenitors of

to establish the tamoxifen dose suitable for clonal labeling.

(DDC)4-diet treatment, mice fed with a choline-deficient ethi-

was observed after 3,5-diethoxycarbonyl-1,4-dihydrocollidine

-generation. They demonstrated no contribution of Hnf1

to hepatocytes was detected (7). Although no contribution

was observed after 3,5-diethoxycarbonyl-1,4-dihydrocollidine

beyond a negligible frequency (8). In fact, they failed to detect

derived from biliary epithelial cells or mesenchymal liver cells

hypothesis that there is virtually all new hepatocytes come from preexist-

to the Cre-expressing adenovirus and also the duration of the Cre-expressing virus in hepa-

tocytes, the virus-mediated lineage-tracing data should be interpreted with caution. We, therefore, generated a hepatocyte-inducible tracing mouse line and directly labeled hepa-

tocytes by tamoxifen induction and traced the change of hepatocyte-labeling efficiency under multiple liver injury condi-

Our studies demonstrated that hepatocytes, not other kinds of liver cells, act as the main source for hepatocyte replenishment and regeneration in adult liver after different types of injury.

Results

Generation and characterization of Alb-DreER mouse line

Albumin is synthesized in the liver and functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a role in stabilizing extracellular fluid volume (13, 14). During liver development, albumin is specifically expressed in liver hepatoblasts (progenitors of both biliary epithelial cells (BECs) and hepatocytes). Its expression is restricted to mature hepatocytes in adult stage (15). We, therefore, utilize the Alb gene to generate a genetic tool for inducible lineage tracing of hepatocytes. We first generated Alb-DreER by knocking a cDNA encoding DreER T2 into the last coding exon of Alb using CRISPR/Cas9 through homologous recombination. This knock-in strategy leads to simultaneous expression of DreER and endogenous albumin. Dre is a site-specific recombinase that targets the rox site, and similar to Cre-loxP, Dre-rox recombination has also been used for genetic lineage tracing (16–18). By crossing Alb-DreER with the R26-RSR-ttdTomato reporter line (16, 17), we generated Alb-DreER;R26-RSR-ttdTo-

mato mice and treated them with tamoxifen for induction of Dre-rox recombination (Fig. 1A). Dre-rox recombination will remove rox-flanked transcriptional Stop cassette, leading to ttdTomato expression and cell labeling (Fig. 1B). This genetic labeling is heritable and irreversible so that all the descendants of Alb-expressing cells will permanently express the genetic marker tdTomato. In adult Alb-DreER;R26-RSR-ttdTomato mice collected 4 days after tamoxifen induction, we observed Alb-DreER efficiently labeled cells in liver (Fig. 1C). Immunostaining for the genetic marker tdTomato, the biliary epithelial cell marker CK19, and hepatocyte marker HNF4a on liver sections showed that Alb-DreER labeled almost all hepatocytes but not BECs (Fig. 1D). To detect the leakiness of DreER, we collected the liver of Alb-DreER;R26-RSR-ttdTomato mice that did not receive tamoxifen. Immunostaining for tdTomato and HNF4a on liver sections shows sparse tdTomato + hepatocytes (Fig. 1D), indicating that a high labeling percentage of hepatocytes after tamoxifen induction is unlikely due to leakiness of DreER. Immunostaining for tdTomato and other non-hepatocyte lineage markers showed that Alb-DreER did not label Sox9 + or EpCAM + biliary epithelial cells, Desmin + hepatic stellate cells, PDGFRα + fibroblasts, VE-cadherin + vascular endothelial cells, or aSMA + smooth muscle cells in the liver (Fig. 1E). Quantification data showed that 99.64 ± 0.15% of hepatocytes were tdTomato + at 1 week after tamoxifen treatment. At 6 weeks after tamoxifen treatment, the percentage of tdTomato + hepatocytes remained similar to that of 1 week (99.54 ± 0.19%, n = 4) (Fig. 1F). Taken together, our results demonstrated that Alb-DreER efficiently and specifically labels...
hepatocytes in adult mouse liver (Fig. 1G), and this genetic tool could be utilized to study the cell fate of hepatocytes after liver injuries.

**Hepatocytes are derived from pre-existing hepatocytes after CCl4 injury**

To test whether hepatocytes arise from progenitor cells after chronic liver injury, we labeled almost all hepatocytes using \textit{Alb-DreER;R26-RSR-tdTomato} mice to determine if most hepatocytes after injury are derived from pre-existing labeled hepatocytes. By using and-chase strategy, we reasoned that if unlabeled hepatic progenitor cells contribute to new hepatocytes after injury, these new hepatocytes would be unlabeled. If the number of unlabeled hepatocytes from progenitor cells is substantial, it would significantly dilute the labeling percentage of hepatocytes. If their contribution is negligible, we may not
detect significant dilution of the labeled hepatocytes. We could, therefore, infer from these data that new hepatocytes are mainly derived from pre-existing hepatocytes or hepatic precursor cells.

Next, we used this pulse-and-chase strategy to analyze the percentage of labeled hepatocytes during the liver regeneration after different types of injury models. The Alb-DreER;R26-RSR-tdTomato mice were treated with CCl4 to induce chronic injury (Fig. 2A). Shortly after CCl4 treatment, we found a significant loss of hepatocytes in the pericentral region compared with fairly normal condition of hepatocytes in the periportal region (Fig. 2B). During chronic injury, periportal hepatocytes will migrate and compensate for the loss of pericentral hepatocytes (19). Sirius red staining confirmed a significant fibrosis in CCl4-treated liver compared with oil-treated liver (Control) or liver collected at 3-weeks’ recovery after injury (Recovery) (Fig. 2C). Whole-mount epifluorescence view of livers from different groups shows no significant dilution of fluorescence signal after liver injury (Fig. 2D). Immunostaining for tdTomato, CK19, and HNF4α on liver sections shows almost all hepatocytes in injured and recovered groups are tdTomato + . F, whole-mount bright-field or fluorescence view of CCl4 injured livers from Alb-DreER;R26-RSR-tdTomato mice without tamoxifen treatment (No Tam). G, immunostaining for tdTomato and CK19 on liver section shows sparse tdTomato + hepatocytes. H, quantification of tdTomato + hepatocytes in different groups. n.s., non-significant; n = 4. Scale bars, 100 µm in B, E, and G; 200 µm in C; 500 µm in D; 1 mm in F. Error bars, S.E.
hepatocytes (Fig. 2E). To exclude that the high labeling efficiency in injured liver is not due to leakiness of DreER, we induced CCl4 in mice that did not receive tamoxifen (No Tam). We detected very sparsely labeled hepatocytes (Fig. 2, F and G), indicating that a high labeling percentage of hepatocytes before and after injury is unlikely due to leakiness of DreER. Quantification of HNF4α/H9251 hepatocytes showed that there is no difference in the percentage of tdTomato+ hepatocytes between control, injury, and recovery groups (99.60 ± 0.11% versus 99.38 ± 0.19% versus 99.34 ± 0.075%, respectively, n = 4, Fig. 2H). The percentage of “leaky” tdTomato− hepatocytes is negligible by quantification (Fig. 2H). Taken together, these results demonstrated that almost all hepatocytes in the injured liver are derived from pre-existing hepatocytes. Hepatic progenitor cells, if present, minimally contributed to new hepatocytes after injury.

Hepatocytes are derived from pre-existing hepatocytes after DDC injury

We further examined the contribution of pre-existing hepatocytes to newly generated hepatocytes in DDC-supplemented diet-induced liver damage models. The Alb-DreER;Rosa26-RSR-tdTomato mice were induced cholestatic injury with a DDC-containing diet (Fig. 3A). Sirius red staining of liver sections showed excessive fibrosis after injury (Fig. 3B). Whole-mount epifluorescence images of liver samples showed no appreciable change of the efficiency of hepatocyte labeling after injury and recovery. More CK19+ BECs were noted after injury, indicating ductal reaction in DDC-induced liver injury model. E, quantification of the percentage of tdTomato+ hepatocytes in different groups. n.s., non-significant; n = 4. Scale bars: 200 μm in B; 1 mm in C (upper) and 500 μm in C (lower); 100 μm in D. Error bars, S.E.

Hepatocytes are derived from pre-existing hepatocytes after CDE injury

We next fed Alb-DreER;Rosa26-RSR-tdTomato mice with a CDE diet to induce liver injury (Fig. 4A). Sirius red staining showed significant fibrosis after CDE treatment (Fig. 4B). Whole-mount fluorescence showed that there is no significant dilution of tdTomato+ signal after injury (Fig. 4C). Immuno-
staining for tdTomato, CK19, and HNF4α showed an increased number of CK19+ BECs cells after injury, indicating ductal reaction. However, the labeling efficiency of hepatocytes still remains high in CDE liver compared with control or recovery (Fig. 4D). Quantification data confirmed that there is no significant difference of the tdTomato+ hepatocyte percentage between control, CDE, and recovery groups (99.58 ± 0.071% versus 99.46 ± 0.098% versus 99.48 ± 0.23%, respectively, n = 4; Fig. 4E). Taken together, these data showed that pre-existing hepatocytes are the major source for new hepatocytes after CDE-induced liver injury.

Hepatocytes are derived from pre-existing hepatocytes after α-naphthyl-isothiocyanate (ANIT) injury

Additionally, we fed Alb-DreER;Rosa26-RSR-tdTomato mice with an ANIT diet, another cholestatic liver injury model (Fig. 5A). Similarly, we used Sirius red staining to confirm the success of liver damage according to inflammatory infiltration and parenchymal necrosis (Fig. 5B). A whole-mount fluorescence view of livers showed no appreciable change in the frequency of hepatocyte labeling after injury. (Fig. 5C). Immunostaining for tdTomato, CK19, and HNF4α on liver sections showed no appreciable change in the frequency of hepatocyte labeling after injury. E, quantification of tdTomato+ hepatocytes in different groups. n.s., non-significant; n = 4. Scale bars, 200 μm in B; 1 mm in C (upper) and 500 μm in C (lower); 100 μm in D. Error bars, S.E.

Hepatocytes are derived from pre-existing hepatocytes after partial hepatectomy (PHx)

As a control, we performed mice surgeries to test the changes of adult hepatocytes. We designed 2/3 PHx using Alb-IRES-DreER;Rosa26-RSR-tdTomato mice and collected livers at different times (Fig. 6A). Sirius red staining showed that there was no fibrosis after PHx, indicating that the surgery is successful, and no other additional injuries were induced (Fig. 6B). Collecting livers from PHx mice proved the size became much larger than sham mice, demonstrating that the extent of hepatocyte proliferation is directly proportional to the amount of resected liver tissue (Fig. 6C). Immunostaining for tdTomato, CK19, and HNF4α showed that there is also no change in the tdTomato labeling index in both the short-time and long-time recovery (Fig. 6D). Quantification data showed that there is no significant difference of the percentage of tdTomato+ hepatocytes in this three groups (Fig. 5D).
between sham and recovery groups (99.55 ± 0.21% versus 99.46 ± 0.25% versus 99.52 ± 0.18%, respectively, n = 4; Fig. 6E).

**Hepatocytes are derived from pre-existing hepatocytes after bile-duct ligation (BDL) injury**

Next, we operated on the Alb-DreER;Rosa26-RSR-tdTomato mice with bile BDL (Fig. 7A). Sirius red staining showed that there was serious fibrosis after BDL, indicating that the surgery is performed successfully (Fig. 7B). Collecting livers from sham mice or BDL-treated mice proved the severe bile duct injury (Fig. 7C). Immunostaining for tdTomato, CK19, and HNF4α showed that there is also no change in the tdTomato labeling percentage after mice recovery (Fig. 7D). Quantification data showed that there is no significant difference of the percentage of tdTomato+ hepatocytes between sham and recovery groups (99.58 ± 0.14% versus 99.60 ± 0.089%, respectively, n = 4; Fig. 7E).

**Discussion**

In this study we used lineage tracing approaches to test the source of hepatocytes after toxin- or surgery-induced liver damage and repair/regeneration. We genetically labeled albumin-expressing hepatocytes with high efficiency and specificity and found that there was no detectable change in labeling proportion with six mentioned types of injury in our research. Almost all hepatocytes were labeled after injury, similar to those labeled before injury. Taken together, our genetic lineage tracing data suggest that non-hepatocyte populations are unable to contribute significantly to hepatocyte neogenesis during liver repair and regeneration.

Recent findings have recrudesced a long-term debate about whether hepatocytes or facultative stem cells, also known as “oval cells” or “atypical ductal cells,” are the main source of new hepatocytes in liver regeneration. It has been proposed that atypical ductal cells are special stem cells that are based on *in vitro* studies and cellular transplantation assays (4, 20). Overall, *in vitro* and cell-transplantation studies are able to reflect the potential of a certain type of cell under the established experimental conditions, whereas lineage-tracing studies prefer to provide insights into cell fate *in vivo* without manipulating cells by isolation, culture, and transplantation.

Indeed, the stem-cell paradigm prevails in many adult tissues. In the mammalian liver the stem-cell paradigm has become a heated debate based on controversial lineage studies, as stem cells are reported to differentiate into new hepatocytes after injury. Genetic lineage-tracing studies based on Cre-loxP systems (Hnf1β, Sox9, Osteopontin, etc.) supported that resident liver progenitor cells contribute to new hepatocytes after...
injury and during liver regeneration (3, 7, 21). Recent lineage-tracing studies suggested that adult hepatocytes are generated by self-duplication rather than stem cell differentiation (10, 22). Almost all of these previous studies used adeno-associated virus (AAV) for expression of Cre recombinase in hepatocytes (10, 22). A popular in vivo reprogramming strategy is delivery of the transcription factors to the targeted cells, which is thought efficient and safe (23, 24). The exploration of used delivery vectors is particularly important. Different AAV capsids have various transducing efficiency for cell types. Most researchers used a replication-incompetent, recombinant adeno-associated virus serotype 2/8 expressing Cre recombinase driven by the hepatocyte-specific promoter (thyroid hormone-binding globulin, AAV8-TBG-Cre) (10). This transduction is highly specific (6, 25). Moreover, this labeling is efficient, as the majority of hepatocytes are genetically marked when mice are infected (10), with no labeling of non-hepatocytes (6). However, the efficiency of this labeling method depends on the doses of vectors that are not controlled very well (26). Meanwhile, the duration of virus-mediated labeling of hepatocytes was unknown. It is also unclear if excessive virus would have any positive or negative influence on the proliferation or function of hepatocytes. The effect of liver injury models on the labeling efficiency and specificity induced by AAVs was also obscure. In our study we used genetic lineage labeling tools Alb-DreER to efficiently label almost all of the hepatocytes to trace the changes of hepatocyte labeling after liver injuries. The pulse-and-chase strategy showed no significant change of hepatocyte labeling by different injury models, suggesting minimal, if any, contribution of facultative stem cells to new hepatocytes. This is by far the first knock-in model to systematically address if pre-existing hepatocytes are the main, if not exclusive, source of new hepatocytes after injury. Our data support the view that new adult hepatocytes arise from pre-existing hepatocytes not only after partial hepatectomy and bile-duct ligation but also in the setting of toxin injuries as well, which is consistent with previous reports (10, 27). As hepatocytes have the remarkable ability of proliferation (28) and can also differentiate into biliary cells on injury (5, 6, 29), hepatocytes themselves appear to constitute the facultative progenitor cell compartment of the liver.

In addition, the novel tool we generated is also useful for a more sophisticated study of different populations, as Cre-loxP and Dre-rox are two orthogonal systems that could be used to genetically target two different cell populations simultaneously in vivo. Furthermore, the precision of the broadly using Cre-loxP system would be improved when it is combined with the Dre-rox system for genetic targeting. Recently, we used the intersectional genetics based on both Cre-loxP and Dre-rox
systems and found that some liver vasculature originates from endocardial cells in development (18), indicating the unique property of Dre-rox system of integrating into the widely used Cre-loxP system. Likewise, one quick application of Alb-DreER in a liver study, for example, is to combine it with Sox9-CreER to more precisely trace Sox9\(^+\)/H11001Alb\(^+\)/H11001-hybrid hepatocytes (30) without targeting of Sox9\(^+\)/H11001Alb\(^+\)/H11002 biliary epithelial cells. Therefore, the Alb-DreER tool generated in this study not only resolves the previous controversy by an in vivo genetic study without depending on excessive virus infection, but it also provides an alternative new mouse tool to the field, facilitating more precise genetic manipulation of hepatocytes in vivo in the future studies.

**Experimental procedures**

**Mice**

All animal studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. Mice were maintained on a C129/C57BL6/J;ICR mixed background. Alb-DreER was generated by CRISPR/Cas9 through homologous recombination. A cDNA encoding IRES-DreERT2 was inserted in-frame with the translational termination codon of the albumin gene. The chimeric mice positive for targeted ES cells were germ line transferred to F1 generation and bred on a background.

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**Figure 7. Hepatocytes were derived from pre-existing hepatocytes after BDL injury.** A. experimental strategy for tamoxifen treatment (Tam) and tissue analysis at different time points after Tam. B. Sirius red staining of liver sections from Sham or BDL-injured mice. R2W, recovery 2 weeks after BDL. C. whole-mount fluorescence view of Alb-DreER;R26-RSR-tdTomato liver at Sham or BDL injury. D, immunostaining for tdTomato, CK19, and HNF4\(\alpha\) on liver sections shows liver histology after BDL injuries with no appreciable change in the frequency of hepatocyte labeling. E, quantification of tdTomato\(^+\) hepatocytes in different groups. n.s., non-significant; n = 4. Scale bars: 200 \(\mu\)m in B; 1 mm in C (upper) and 500 \(\mu\)m in C (lower); 100 \(\mu\)m in D. Error bars, S.E.
C57BL/6;ICR background. Ai66 (Rosa26-Rox-Stop-Rox-LoxP-Stop-LoxP-tdTomato) was reported previously (31). The Rosa26-Rox-Stop-Rox-tdTomato (Rosa26-RSR-tdTomato) mouse line was generated by crossing ACTB-Cre (32) with Ai66 to excise the LoxP-flanked Stop cassette, and ACTB-Cre was not passaged to the subsequent generation. Rosa26-RSR-tdTomato (16, 17) was responsive to Dre but not Cre recombinase. The Alb-DreER line was generated by Shanghai Biomodel Organism Science and Technology Development Co., Ltd. All experimental mice were maintained on a C57BL/6;ICR background. Tamoxifen (Sigma, T5648) was dissolved in corn oil and administered to mice twice, cleared in xylene, and mounted with resinous medium. Images were obtained on an Olympus fluorescence microscope (BX53), a Zeiss stereomicroscope (AXIO Zoom. V16), a Zeiss confocal laser scanning microscope (LSM510), and an Olympus confocal microscope (FV1200).

Genomic PCR

Genomic DNA was prepared from mouse toes or tail. Tissues were lysed by incubation with proteinase K overnight at 55 °C followed by centrifugation at maximum speed for 8 min to collect supernatant with genomic DNA. DNA was precipitated with isopropanol and washed with 70% ethanol. All mice were genotyped with specific primers that distinguish knock-in allele from wild-type allele. For the wild-type allele.

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Genetic lineage trace the hepatocyte neogenesis


