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

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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 cholangiocyttes 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).

However, recent studies using genetic lineage-tracing experiments suggested that oval cells contribute minimally to hepato-
Genetic lineage trace the hepatocyte neogenesis

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 (precursors 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 

and co-workers (8) injected into reporter mice an adenovirus vector expressing Cre from the transthyretin promoter (AAV8-Ttr-Cre) that afforded specific and efficient reporter gene activation in hepatocytes but did not label BECs, stellate cells, macrophages, or endothelial cells in livers. In contrast to previous studies, they failed to detect hepatocytes derived from biliary epithelial cells or mesenchymal liver cells beyond a negligible frequency (8). In fact, they failed to detect hepatocytes that were not derived from pre-existing hepatocytes. Their findings argue against liver stem cells or other non-hepatocyte cell types, providing a backup system for hepatocyte regeneration in chronic liver injury models (8). Grompe and co-workers (9) also used clonal tracing strategy to further research the liver progenitors issue. The biliary or nonparenchymal compartments were traced en masse, which precludes the identification of clonal relationships between hepatocytes and ductal progenitors. It has been reported that tamoxifen can induce “ectopic” expression of ductal markers in hepatocytes (11), and biliary transcription factors are expressed in normal hepatocytes (12). Grompe and co-workers suggested that a clonal labeling strategy is needed to directly identify the origin of hepatocyte precursor cells in liver repair. They used Sox9-CreER 

and regenerate in adult liver after different types of injury. 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.

Genetic lineage trace the hepatocyte neogenesis

| Genes and proteins | Abbreviations |
|-------------------|---------------|
| Sox9              | Sox9          |
| Hnf1β             | Hnf1β         |
| Hnf1              | Hnf1          |
| ALB               | Alb           |

4 The abbreviations used are: DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; BEC, biliary epithelial cell; CDE, choline-deficient ethionine-supplemented; ANIT, α-naphthylisothiocyanate; PHx, partial hepatectomy; BDL, bile-duct ligation; AAV, adenovirus; VE-cadherin, vascular endothelial cadherin.
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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 Alb-DreER;Rosa26-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 liver samples 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+ (*Fig. 2E and 2F*). By CCl4 treatment, we did not detect any BECs derived from tdTomato+.
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 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 livers showed no significant drop of tdTomato+ signals in DDC-induced injury group compared with control or recovery groups (Fig. 3C). Immunostaining for tdTomato, CK19, and HNF4α on liver sections shows 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. 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.

Figure 4. Hepatocytes were derived from pre-existing hepatocytes after CDE 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 Control or CDE-treated mice. C, whole-mount fluorescence view of Alb-DreER;R26-RSR-tdTomato liver from Control, CDE, or recovery groups. D, 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 these three groups (Fig. 5D). Quantification data showed that there is no significant difference of the percentage of tdTomato+ hepatocytes between control, ANIT, and recovery groups (99.61 ± 0.081% versus 99.56 ± 0.14% versus 99.47 ± 0.18%, respectively, n = 4; Fig. 5E). Taken together, these data demonstrated that pre-existing hepatocytes are the major origin for new hepatocytes in ANIT-induced liver injury model.
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-H11001 Alb-H11001-hybrid hepatocytes (30) without targeting of Sox9-H11001 Alb-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.

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 HNF4α 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 μm in B; 1 mm in C (upper) and 500 μm in C (lower);100 μm in D. Error bars, S.E.

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;JCR mixed background. Alb-DreER was generated by CRISPR/Cas9 through homologous recombination. A cDNA encoding IRES-DreER 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
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 at the indicated time. Adult mice received 0.1–0.2 mg of tamoxifen/g of mouse body weight by oral gavage.

Genomic PCR

Genomic DNA was prepared from mouse toes or tail. Tissues were lysed by incubation with protease K overnight at 55 °C followed by centrifugation at maximum speed for 8 min to obtain supernatant with genomic DNA. DNA was precipitated twice, cleared in xylene, and mounted with resinous medium. All experimental mice were maintained on a C57BL/6;ICR background. Genomic DNA was prepared from mouse toes or tail. Tissues were lysed by incubation with protease K overnight at 55 °C followed by centrifugation at maximum speed for 8 min to obtain supernatant with genomic DNA. DNA was precipitated twice, cleared in xylene, and mounted with resinous medium.

Injury model

For the CCL4-induced chronic injury model, CCL4 was dissolved at 1:3 in corn oil and injected intraperitoneally at a dose of 4 μl/g body weight every 3 days for 10 times (22). Mice were given 0.1% wt/wt DDC (Sigma) PMI Mouse Diet #5015 (Harlan Teklad) for 6 weeks, at which point the diet was changed to regular chow for 2 weeks to allow mice to recover. A choline-deficient diet (FBSH China) supplemented with 0.15% ethionine nine drinking water (Sigma, E5139) was administered for 8 weeks followed by 4 weeks of recovery. ANIT (dissolved in oil, 60 mg/kg) was administered intraperitoneally every 3 days for 10 times followed by 3–4 weeks of recovery. PHx and BDL were performed as described previously (19).

Immunostaining

Immunostaining was performed according to protocols described previously (33). Tissues were dissected in PBS and fixed in 4% paraformaldehyde (Sigma) at 4 °C for 1 h. Afterward, tissues were washed in PBS and dehydrated in 30% sucrose overnight at 4 °C and embedded in OCT (Sakura). 10-μm cryosections were obtained and air-dried afterward at room temperature. For staining, dried sections were washed in PBS and then blocked with 5% normal donkey serum (Jackson ImmunoResearch) and 0.1% Triton X-100 in PBS for 30 min at room temperature. Sections were incubated with the primary antibodies overnight at 4 °C. The following antibodies were used: tdTomato (Rockland, 600-401-379, 1:200), cytokeratin 19 (Developmental Studies Hybridoma Bank, TROMA-III, 1:100), HNF4α (Santa Cruz, sc-6556, 1:100), VE-cadherin (R&D, AF1002, 1:100), Desmin (R & D, AF3844, 1:100), PDGFα (R&D, AF1062, 1:100), EpCAM (Abcam, ab92383, 1:100), Sox9 (Millipore, AB5535, 1:100), αSMA (Sigma, F3777, 1:100). Signals were developed with Alexa fluorescence antibodies (Invitrogen), and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Immunostaining images were acquired by an Olympus fluorescence microscope (BX53), a Zeiss stereomicroscope (AXIO Zoom, V16), a Zeiss confocal laser scanning microscope (LSM510), and an Olympus confocal microscope (FV1200).

Sirus red staining

Sirus red staining was aimed to assess fibrotic tissue formation after chronic injury and was performed as described previously (34). Cryosections were fixed in 4% paraformaldehyde for 15 min, then washed in PBS for 15 min and fixed overnight in Bouins solution (5% acetic acid, 9% formaldehyde, 0.9% picric acid). Subsequently, sections were stained with 0.1% Fast Green (Fisher) for 3 min and incubated in 1% acetic acid for 1 min followed by incubation with 0.1% Sirius red (Sigma) for 2 min. Sections were rinsed with tap water before incubation into the staining solution. Slides were dehydrated in 100% ethanol twice, cleared in xylene, and mounted with resinous medium. Images were obtained on an Olympus microscope (BX53).

Statistics

Data for two groups were analyzed by a two-tailed unpaired Student’s t test, whereas comparison between more than two groups was performed using an analysis of variance followed by Tukey’s multiple comparison test. Significance was accepted when p < 0.05. All data are presented as the mean ± S.E.

Author contributions—Y. W. and B. Z. designed the study, performed the experiments, and analyzed the data. X. H., L. H., W. P., Yan Li, Q. L., Yi Li, L. Z., W. Y., H. Z., and Y.Z. bred the mice, performed the experiments, and analyzed the data. X. H., L. H., W. P., Yan Li, Q. L., Yi Li, L. Z., W. Y., H. Z., and Y.Z. bred the mice, performed the experiments, and provided intellectual input. B. Z. conceived and supervised the study, analyzed the data, and wrote the manuscript.

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