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Regeneration of Liver After Extreme Hepatocyte Loss Occurs Mainly via Biliary Transdifferentiation in Zebrafish

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BACKGROUND & AIMS: The liver has high regenerative capacity, but it is not clear whether most biliary cells (particularly larger cholangiocytes) transdifferentiate into hepatocytes in regenerating liver. We investigated how this process might contribute to liver regeneration in zebrafish. **METHODS:** Zebrafish transgenic lines were generated using the standard I-SceI meganuclease transgenesis technique. Hepatocytes of the *Tg(lfabp:mCherry-NTR)*^{cq2} animals were ablated by the administration of metronidazole. We investigated transdifferentiation of biliary cells to hepatocytes and expression of markers using whole mount antibody staining, fluorescent in situ hybridization, and Cre/loxP-based genetic lineage tracing analyses. The role of biliary cells in hepatocyte regeneration was explored using zebrafish larvae with defects in biliary cell development. **RESULTS:** After extreme loss of hepatocytes, nearly all the biliary cells steadily lost their tubular morphology, proliferated, and expressed hepatocyte-specific markers. Cre/loxP-based inducible lineage tracing showed that new hepatocytes mainly arose from transdifferentiation of biliary cells; this process required Notch signaling and, in turn, activation of Sox9b in cholangiocytes. Activation of early endoderm and hepatoblast markers in most of the cholangiocytes indicated that biliary transdifferentiation includes a step of dedifferentiation into a bipotential intermediate. Defects in development of biliary cells impaired hepatocyte regeneration. **CONCLUSIONS:** Using our zebrafish liver regeneration model, we found that biliary cells can transdifferentiate into hepatocytes and are the major contributors to hepatocyte regeneration after extreme hepatocyte loss.

Keywords: Liver Damage; Animal Model; Cholangiocyte Transdifferentiation; Developmental Signals.

Regeneration after surgical removal of a liver mass (partial hepatectomy) is mainly achieved by proliferation of residual, healthy hepatocytes. However, in cases of extreme hepatocyte loss or end-stage chronic liver disease, most of the hepatocytes become depleted or lose their proliferative capacity. Thus, identification of in vivo cell origins other than residual hepatocytes that contribute to liver regeneration is of great scientific and clinical interest.[1]

Cell transdifferentiation has been identified as one of the important sources of organ regeneration. One classic example is the regenerating lens of the newt. After dissection of the lens, pigmented epithelial cells lose their pigmentation and change the cell shape while dedifferentiating, proliferating, and differentiating into mature cells of the lens.[2] Thus, transdifferentiation is believed to be associated with 2 steps; mature cells dedifferentiate into an intermediate cell type, which then differentiates into new mature cell lineages.[3–6] In the context of the liver, transdifferentiation has been identified both in vitro and in vivo. In vitro, combinations of transcription factors can convert mouse fibroblasts to induced hepatocyte-like cells, which are able to repopulate injured livers and restore liver functions.[7,8] In vivo, hepatocytes can transdifferentiate into biliary epithelial cells,[9–12] which is dependent on phosphatidylinositol 3-kinase.[13] In reverse, hepatocytes can derive from intrahepatic stem cells that are subpopulations of small cholangiocytes lining the Hering canal and ductules.[14] However, whether the majority of biliary cells, particularly larger cholangiocytes, have the potential to transdifferentiate into hepatocytes and whether this transdifferentiation takes place in vivo and contributes to hepatocyte regeneration remain unknown.

The Notch signaling pathway, downstream of which the transcriptional factor Sox9 associates, is required for biliary development.[15–19] In the context of liver regeneration, nuclear translocation of the intracellular cytoplasmic domain of Notch increases after partial hepatectomy.[20] The injury-induced oval cell response and hepatocyte-to-biliary reprogramming require Notch.[10,21] Notch activation is also critical for the conversion of hepatocytes to biliary lineage during the onset, malignancy, and progression of intrahepatic cholangiocarcinoma.[22] In adults, Sox9 is expressed in a subpopulation of biliary epithelia. Whether these Sox9-expressing cells are the cell sources of liver homeostasis and regeneration as well as the role of *sox9* remain unclear.[23–27]

Zebrafish appear to be a powerful system to study organ regeneration, such as the heart and the kidney.[28–31] The zebrafish larvae are ready to feed at 5 days postfertilization (dpf), after which the liver is fully operational as in adults, including bile production, glycogen storage, and lipid

Abbreviations used in this paper: **DMSO, dimethyl sulfoxide; dpf, days postfertilization; hpt, hours posttreatment; MTZ, metronidazole; NTR, bacterial nitroreductase; 4-OHT, 4-hydroxytamoxifen; PBS, phosphate-buffered saline; PBST, 1% Triton X-100 in phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde.**

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homeostasis.[32] Therefore, similar to adults, zebrafish larvae are suitable for study of liver regeneration after 5 dpf. In zebrafish, the bacterial nitroreductase (NTR) and its substrate metronidazole (MTZ) have been successfully introduced to induce conditional targeted cell ablation for regeneration studies.[33]

To address the in vivo cell origins of hepatocyte regeneration, particularly in cases of extreme hepatocyte loss, we generated *Tg(lfabp:mCherry-NTR)*^{cq2} and *Tg(lfabp:Dendra2-NTR)*^{cq1} zebrafish transgenic lines. Using these lines, our study shows that the majority of biliary cells display the potential to transdifferentiate into hepatocytes, and this transdifferentiation becomes the major in vivo contributor to hepatocyte regeneration after extreme hepatocyte loss.

Materials and Methods

Zebrafish Strains

Zebrafish (*Danio rerio*) of the AB genetic background *Tg(lfabp:Dendra2-NTR)*^{cq1} abbreviated as *Tg(lfabp:DenNTR)*^{cq1}, *Tg(lfabp:mCherry-NTR)*^{cq2} abbreviated as *Tg(lfabp:mChNTR)*^{cq2}, *Tg(hsp70l:loxP-DsRed-loxP-GFP)*^{cq3}, *Tg(lfabp:loxP-STOP-loxP-DsRed2)*^{cq4}, and *Tg(lfabp:DsRed)*, *Tg(EPV.Tp1-Mmu.Hbb:eGFP)*^{um14} abbreviated as *Tg(Tp1:eGFP)*^{um14}, and *Tg(Tp1bglob:CreER*^{T2}*)*^{jh12} abbreviated as *Tg(Tp1:CreER*^{T2}*)*^{jh12} transgenic lines, *cq5* mutant, and *sox9b*^{fh313} mutant lines were raised and maintained under standard laboratory conditions according to institutional animal care and use committee protocols.

BODIPY Assay and Periodic Acid–Schiff Staining

Four hours before live imaging, larvae were fed with BODIPY FL C5 (Invitrogen, Grand Island, NY) as previously described.[34] Periodic acid–Schiff staining was performed as previously described.[8]

Cell Sorting, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction

The livers of 100 *Tg(Tp1:eGFP; lfabp:mChNTR)* transgenic larvae were dissected, and hepatocytes were dissociated and sorted as previously described.[35] Quantitative real-time polymerase chain reaction was performed for *hhex*, *foxa3*, *sox9b*, and *eGFP* using the FastStart Universal SYBR Green Master (Roche, Indianapolis, IN), normalized by transcriptions of *β-actin*. Primer sequences are available on request.

In Situ Hybridization, Antibody Staining, and Imaging

Whole mount in situ hybridization was performed as previously described[36] using the *sox9b*, *hhex*, *and hes5* probes. Whole mount antibody staining was performed as previously described[35] using antibodies against Dendra2 (1:100; Evrogen, Moscow, Russia), green fluorescent protein (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), 2F11 (1:1000; Abcam, Cambridge, MA), Alcam (1:50; Zirc, Eugene, OR), DsRed2 (1:100; Santa Cruz Biotechnology), mCherry (1:100; Abcam), and Foxa3 (1:100; Anaspec, Fremont, CA).

Whole mount in situ hybridized larvae were imaged using a SteREO DiscoveryV20 microscope equipped with AxioVision Rel 4.8.2 software (Carl Zeiss, Jena, Germany). Antibody stained and live larvae were imaged using ZEN2010 software equipped on an LSM780 confocal microscope (Carl Zeiss).

Treatment With MTZ, DAPT, or DAPM

The larvae at 6 dpf were incubated with 10 mmol/L MTZ (Sigma, St. Louis, MO) in 0.2% dimethyl sulfoxide (DMSO) for 24 hours. Then, larvae were washed 3 times and recovered in egg water.

A total of 50 μmol/L DAPT working solution was refreshed every 24 hours. Control larvae were incubated in 0.5% DMSO in egg water. For DAPT pretreatment experiments, the *Tg(lfabp:-DenNTR)* transgenic larvae was pretreated with DAPT from 74 to 125 hours postfertilization (hpf) and then washed with egg water. Twelve to 16 hours later, the larvae were subjected to treatment with MTZ. For DAPT treatment experiments, the larvae were incubated with 50 μmol/L DAPT and 10 mmol/L MTZ simultaneously for 24 hours. After withdrawal of MTZ, incubation with DAPT was continued until fixation.

A total of 0.2 mol/L DAPM (Sigma) stock solution was in 100% ethanol, and 80 μmol/L DAPM working solution was refreshed every 24 hours. Control larvae were incubated in 0.04% ethanol in egg water. Larvae were incubated with 80 μmol/L DAPM and 10 mmol/L MTZ simultaneously for 24 hours. After withdrawal of MTZ, incubation with DAPM was continued until fixation.

Temporal Control of CreER^{T2} Activities

4-Hydroxytamoxifen (4-OHT; Sigma) was dissolved in 100% ethanol to prepare a stock concentration of 10 mmol/L. Larvae were incubated with 5 μmol/L 4-OHT in egg water at 28°C for 24 hours, followed by 3 washes with fresh egg water and incubation for another 12 hours before treatment with MTZ.

5-ethynyl-2′-deoxyuridine (EdU) Labeling and Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assays

The Click-iT Kit (Invitrogen) was applied for S-phase labeling according to the manufacturer's instructions. For terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay, larvae were fixed overnight in 2% formaldehyde in 0.1 mol/L piperazine-*N,N′*-bis(2-ethanesulfonic acid), 1 mmol/L $MgSO_4$, 2 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, pH 7.0, at 4°C overnight followed by skin removal. Larvae were assayed using the In Situ Cell Death Detection Kit, TMR Red (Roche), and the In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions.

Combination of Fluorescent In Situ Hybridization and Antibody Staining

Larvae were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C overnight, followed by incubation in 100% methanol at −20°C for 24 hours. After treatment of 3% H_2O_2 in methanol, larvae were serially transferred into 75%, 50%, 25%, and 0% methanol in PBST (1% Triton X-100 in PBS). Then, the larvae were refixed in 4% PFA and the skins were manually removed. After washing 5

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