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Chemical Screening Using a Zebrafish Model for Liver Progenitor Cell-Driven Liver Regeneration

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Abstract

Following massive hepatocyte ablation in zebrafish, biliary epithelial cells can extensively give rise to hepatocytes through liver progenitor cells (LPCs). The zebrafish liver injury model is an important system to elucidate the molecular mechanisms underlying LPC-driven liver regeneration. Here, we describe a chemical screening method using the zebrafish model for identifying small molecules that can modulate LPC-driven liver regeneration.

Keywords

Nitroreductase; Biliary epithelial cells; Hepatocyte ablation; Chemical screen; Liver progenitor cells

1 Introduction

The liver is a highly regenerative organ: it can recover even after losing 70% of its mass [1]. However, after prolonged or severe liver injury, as observed in the advanced stages of chronic and acute liver diseases, the liver loses its ability to regenerate through self-replication of hepatocytes [2, 3]. This continuing damage in combination with defective hepatocyte proliferation stimulates excessive inflammation and subsequent wound healing mechanisms, including fibrotic/cirrhotic responses [1–3]. Currently, liver transplantation is the only reliable cure to prolong the life of patients with advanced liver diseases; however, the number of liver donors is not sufficient to match the increasing number of patients [4]. To alleviate this bottleneck and to extend patient lives until transplantation, diverse approaches have been attempted to develop therapeutic options [5–7]. Although there have been technical improvements, further progress is still needed for these therapeutics to be considered as reliable options worthy of testing in clinical trials.

One attractive approach that has recently garnered attention is to promote the differentiation of activated liver progenitor cells (LPCs) in diseased livers into either hepatocytes or biliary epithelial cells (BECs) [3, 8]. Given the prevalence of LPCs in diseased human livers [2, 9], this approach becomes more promising due to recent findings in mice that activated BEC-derived LPCs are a key source of new hepatocytes in various liver injury settings with defective hepatocyte proliferation [9, 10]. While these achievements are encouraging to elucidate the molecular mechanisms underlying LPC-driven liver regeneration and to

identify drug targets that can augment this process, the mouse is not an ideal model animal for large-scale chemical screening due to its high cost and time constraint.

Given the ease of chemical screening in zebrafish embryos and larvae [11], we have established a zebrafish liver injury model for LPC-driven liver regeneration by generating a transgenic line, *Tg(fabp10a:CFP-NTR)*, that provides a genetic means to ablate differentiated hepatocytes [12]. The transgenic fish expresses bacterial nitroreductase (*NTR*) enzymes fused with cyan fluorescent proteins (*CFP*) specifically in hepatocytes due to the hepatocyte-specific *fabp10a* promoter. Since *NTR* converts the non-toxic prodrug metronidazole (Mtz) into a cytotoxic drug, treating the fish with Mtz ablates only *NTR*-expressing hepatocytes. Following massive hepatocyte ablation, BECs dedifferentiate into LPCs and subsequently differentiate into hepatocytes [12]. Compared to the aforementioned mouse model, this zebrafish model is advantageous for chemical screening to identify small molecules that affect LPC-driven liver regeneration [13]: (1) the entire liver regeneration assay takes only 6 days, (2) the regeneration process is homogeneous with very small variability among animals, and (3) hundreds of animals can be manipulated simultaneously. The zebrafish assay allows for screening ~30 compounds per week. Here we describe a detailed protocol for chemical screening using the zebrafish hepatocyte ablation model.

2 Materials

1. *Tg(fabp10a:CFP-NTR);Tg(fabp10a:DsRed)* adult fish.
2. Mating tanks: large aquaculture tanks with dividers.
3. Fine plastic mesh strainer (tea strainer).
4. Squeeze bottle.
5. Petri dishes: 100 × 15 mm and 60 × 15 mm.
6. Egg water: 0.3 g of sea salts “Instant Ocean” in 1 L of distilled water supplemented with 0.5-mM CaSO₄.
7. Disposable polyethylene transfer pipettes.
8. Stereomicroscope.
9. 5-mM phenylthiourea (PTU) stock solution: 760 mg of PTU in 1 L of distilled water. Heat the solution to 60 C until completely dissolved.
10. 24.2-mM tricaine (3-amino benzoic acid ethyl ester, also known as MS-222) stock solution: 400 mg of tricaine powder in 100 mL of distilled water, pH 7–7.5.
11. 100% dimethyl sulfoxide (DMSO, molecular grade).
12. Epifluorescence microscope with DsRed and CFP filters.
13. 10-mM Mtz solution: 0.171 g of Mtz in 100 mL of egg water supplemented with 0.2% DMSO and 0.2-mM PTU. Stir rapidly using a magnetic stirrer at room temperature (RT) for 30 min.
14. 12-well tissue culture plates.

15. 15-mL conical tubes.
16. Aluminum foil.
17. 3% methyl cellulose: add 15 g of methyl cellulose into 500 mL of heated egg water (60 °C) and stir with a magnetic stirrer at RT for 20 min.
18. Superfine eyelash with handle (Ted Pella Inc., Prod No. 113).
19. ImageJ software: it can be obtained at <https://imagej.nih.gov/ij/index.html>.
20. Nitrile gloves.
21. System water: water used to raise and maintain zebrafish.

3 Methods

The outline of the screening process is shown in Fig. 1.

3.1 Preparation of Larvae

1. In each mating tank, place *Tg(fabp10a:CFP-NTR);Tg(fabp10a:DsRed)* male (3) and female (3) fish that are separated by sex with a tank divider. To obtain enough embryos for screening, set up five tanks. Keep tanks at 28 °C overnight.
2. On the following morning, replenish the tanks with fresh system water, and remove the dividers. After 1–2 h, collect embryos by straining the water containing eggs with a fine plastic mesh strainer.
3. Rinse the strainer with a fine stream of system water from a squeeze bottle. Transfer embryos into 100-mm petri dishes with ~25 mL of fresh egg water. Keep no more than 100 embryos per dish and incubate them at 28 °C.
4. About 5–6 h later, under a stereomicroscope, remove unfertilized embryos using a transfer pipette. Replenish with fresh egg water and keep at 28 °C overnight.
5. On the next morning around 24-h post-fertilization (hpf), check the embryos under a stereomicroscope, and remove dead or deformed embryos using a transfer pipette. After cleaning, to inhibit embryo pigmentation, add PTU (final concentration: 0.2 mM) into each petri dish, and raise embryos until 3.3 days post-fertilization (dpf) at 28 °C (see Note 1).
6. Anesthetize the embryos by adding tricaine (final concentration: 0.016%) into the petri dishes (see Note 2). Sort CFP and DsRed double-positive larvae under an epifluorescence microscope. Transfer the double-positive embryos into a new 100-mm petri dish. Remove the egg water containing tricaine and wash three times with egg water. Then, add fresh egg water supplemented with 0.2-mM PTU and keep the embryos at 28 °C.

3.2 Mtz and Compound Treatments

1. Prepare 10-mM Mtz solution (see Subheading 2, **item 13**, and Note 3).

2. Prepare the mixture of each compound and Mtz in a 15-mL conical tube: add 6 μ L of each compound solution (*see* Note 4) into 2-mL Mtz solution and vortex (*see* Note 5). For a control group, add 6 μ L of DMSO into 2-mL Mtz solution (*see* Note 6).
3. Dispense 10–15 larvae into each well of 12-well tissue culture plates using a transfer pipette (*see* Note 5). Remove egg water from each well and add 2 mL of Mtz/compound mixture. To prevent the photoinactivation of Mtz and compounds, cover the plates with aluminum foil, and incubate them at 28 C for 36 h.
4. Prepare each compound solution in egg water: add 6 μ L of each compound solution and 4 μ L of DMSO into 2 mL of egg water supplemented with 0.2-mM PTU and vortex. For a control group, add 10 μ L of DMSO into 2 mL of egg water supplemented with 0.2-mM PTU (*see* Note 5).
5. After 36 h of incubation, to immobilize the larvae, add a lower concentration of tricaine (final concentration: 0.004%) (*see* Note 7). Sort out larvae with well-ablated livers (optional, *see* Note 8). Remove the Mtz/compound solution and add 2 mL of egg water. Gently transfer the embryos into a well of a new 12-well plate using a transfer pipette and swirl the plate twice to wash the embryos.
6. Remove the egg water and add 2 mL of the compound-containing egg water. Cover the plates with aluminum foil and incubate at 28 C for 24 h.

3.3 Image Acquisition and Analysis

1. Anesthetize the larvae by adding tricaine (final concentration: 0.016%) into each well of the 12-well plates. Gently transfer the larvae into 60-mm petri dishes filled with 3% methyl cellulose and using a superfine eyelash (*see* Subheading 2, **item 18**); orient the larvae laterally to clearly reveal the liver.
2. Take a picture using an epifluorescence microscope with a DsRed filter (Fig. 2).
3. Once images are acquired, the ImageJ software is used to measure liver area, the integrated density of DsRed, and the mean background fluorescence, which are used to calculate corrected total cell fluorescence (CTCF) using the formula below [14]. CTCF value represents DsRed expression level (Fig. 2c).

CTCF = Integrated density—(selected area $\times$ the mean fluorescence of background readings).

4 Notes

1. PTU is toxic. Use in accordance with appropriate handling guidelines.
2. Tricaine is toxic. Use in accordance with appropriate handling guidelines.
3. Prolonged exposure or increased concentration of Mtz is toxic. Use in accordance with appropriate handling guidelines.

4. The working concentration of each compound can be determined by treating *Tg(fabp10a:CFP-NTR)* larvae in a 96-well plate (three larvae per well) with various concentrations of the compound together with 10-mM Mtz; the maximum tolerated concentration is used for screening.
5. For a well of a 12-well plate, 2 mL is a proper volume (10–15 larvae per well); 5 mL is for a well of a 6-well plate (15–25 larvae per well).
6. Most chemicals are dissolved in DMSO as a concentrated stock. To prevent any chemical hazards, nitrile gloves are recommended during all screening procedures.
7. Tricaine is metabolized in the liver and can be hepatotoxic; to avoid aggravating liver damage in the Mtz-treated larvae, use 1/4–1/5 dose of tricaine.
8. (Optional) Remove larvae with partial ablation by examining them under an epifluorescence microscope with a DsRed filter. Use larvae with a tiny liver, indicating the full ablation of hepatocytes. Caution: be aware that some compounds can suppress NTR/Mtz-induced hepatocyte ablation, preventing the full ablation. Thus, the effect of these compounds on LPC-driven liver regeneration cannot be assessed using the zebrafish hepatocyte ablation model.

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Fig. 1. Cartoon illustrating the entire chemical screening process for identifying compounds that affect LPC-driven liver regeneration. Red circles denote livers

Fig. 2.

Chemical screening identifies SU5416 and U0126 as a negative and a positive regulator of LPC-driven liver regeneration, respectively. (a) Experimental scheme illustrating the stages of Mtz and compound treatments and analysis (arrow). A0h and A36h, ablation for 0 and 36 h, respectively; R24h, regeneration for 24 h. (b) Epifluorescence images showing *fabp10a:DsRed* expression in regenerating larvae at R24h. 2- μ M SU5416 and 100- μ M U0126 were used. Scale bar, 100 μ m. (c) Quantification of *fabp10a:DsRed* expression in the regenerating livers, as shown in (b). One-way ANOVA; *, $P < 0.05$; error bars, $\pm$ SEM