Video Article

Development of an Ethanol-induced Fibrotic Liver Model in Zebrafish to Study Progenitor Cell-mediated Hepatocyte Regeneration

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Abstract

Sustained liver fibrosis with continuation of extracellular matrix (ECM) protein build-up results in the loss of cellular competency of the liver, leading to cirrhosis with hepatocellular dysfunction. Among multiple hepatic insults, alcohol abuse can lead to significant health problems including liver failure and hepatocellular carcinoma. Nonetheless, the identity of endogenous cellular sources that regenerate hepatocytes in response to alcohol has not been properly investigated. Moreover, few studies have effectively modeled hepatocyte regeneration upon alcohol-induced injury. We recently reported on establishing an ethanol (EtOH)-induced fibrotic liver model in zebrafish in which hepatic progenitor cells (HPCs) gave rise to hepatocytes upon near-complete hepatocyte loss in the presence of fibrogenic stimulus. Furthermore, through chemical screens using this model, we identified multiple small molecules that enhance hepatocyte regeneration. Here we describe in detail the procedures to develop an EtOH-induced fibrotic liver model and to perform chemical screens using this model in zebrafish. This protocol will be a critical tool to delineate the molecular and cellular mechanisms of how hepatocyte regenerates in the fibrotic liver. Furthermore, these methods will facilitate potential discovery of novel therapeutic strategies for chronic liver disease *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/54002/

Introduction

Despite the remarkable regeneration capacity of hepatocytes[1], which are the major parenchymal cell type of the liver, chronic liver failure impairs this ability, leading to hepatic progenitor cell (HPC)-dependent regeneration[2].

Chronic liver damage is mainly derived from alcohol abuse, chronic hepatitis C virus (HCV) infection[3] and non-alcoholic fatty liver disease (NAFLD)[4]. It leads to sustained liver fibrosis, which is associated with the accumulation of extracellular matrix (ECM) proteins. Persisting ECM accumulation distorts intact hepatic architecture by forming a fibrous scar tissue[5], subsequently resulting in cirrhosis with high morbidity and mortality. Many attempts have been made to mitigate the fibrotic response mainly by focusing on inhibiting profibrogenic cytokines and activated myofibroblasts[6]. The latter is primarily derived from hepatic stellate cells (HSCs), the principle hepatic non-parenchymal cells responsible for liver scar formation[4]. Nevertheless, regenerative therapies that stimulate endogenous cellular sources including HPCs to regenerate hepatocytes in the presence of sustained fibrogenic insults await further investigation.

Many experimental models of hepatic fibrosis have been described in mammals. Repetitive injection of carbon tetrachloride (CCl_4) has been widely used to induce liver fibrosis in murine and rat models[7]. When combined with a high-fat (HF) diet, alcohol led to a substantial upregulation of profibrogenic gene expression and hepatic fibrosis[8]. While steatosis (lipid accumulation) results from acute alcohol exposure, it makes the liver susceptible to more severe hepatic injury[9].

The zebrafish, *Danio rerio,* has emerged as an invaluable vertebrate model system for studying regeneration. Though other lower vertebrates such as newts and axolotls have a remarkable capacity for regeneration, the zebrafish has advantages over other model systems in regards to the gene manipulation and visualization strategies needed to manipulate potential regenerative factors[10]. The zebrafish also represents an attractive vertebrate model for studying alcoholic liver disease (ALD) by simply adding ethanol (EtOH) to their water. Acute EtOH exposure to larval and adult zebrafish caused hepatic steatosis[11-13]. When adult zebrafish received extended EtOH exposure, collagen deposition was observed with upregulation of fibrosis-related genes[14]. However, a need exists for developing models to study liver regeneration in response to EtOH as a fibrogenic stimulus.

Recently, we developed an EtOH-induced fibrotic liver model in zebrafish[15]. We combined a hepatocyte-specific genetic ablation system with EtOH treatment in larval and adult zebrafish. We generated two transgenic lines, *Tg(fabp10a:CFP-NTR)^{gt1}* and *Tg(fabp10a:mCherry-NTR)^{gt2}*, in which *E.coli* nitroreductase (NTR) are fused to the cyan and mCherry fluorescent protein, respectively, under the control of the hepatocyte-specific *fatty acid binding protein 10a, liver basic* (*fabp10a*) promoter. In this system, NTR converts a nontoxic prodrug metronidazole (MTZ) into a DNA inter-strand cross-linking agent[16], inducing explicit death of hepatocytes. Using this model, we demonstrated that a population of hepatic

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cells, which are responsive to Notch signaling, converted into hepatocytes in the near absence of hepatocytes and in the excess of ECM. We designated these cells as HPCs. Furthermore, through chemical screens, we identified small molecule activators of Wnt signaling and inhibitors of Notch signaling that augment hepatocyte regeneration in the fibrotic liver. Therefore, our fibrotic liver model in zebrafish represents a superb chemical screening system compared to cell culture- or mammalian-based screening system. It is an *in vivo* system with significant cost- and time-saving benefits. Here we describe the detailed procedures for establishing an EtOH-induced fibrotic liver model and for performing chemical screens using this model in zebrafish. Furthermore, time-course analyses were performed to investigate how hepatocyte regeneration occurs in the fibrotic liver. This protocol will provide an invaluable tool to study the mechanisms and strategies of enhancing hepatocyte regeneration in the fibrotic liver.

Protocol

Zebrafish were raised and bred using a standard protocol that meets the criteria of the National Institutes of Health and approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee.

1. Preparation of Solutions

1. Prepare 20 L egg water (interchangeably used with 'embryo medium') to maintain embryonic/larval zebrafish. Dissolve 1.5 g $CaSO_4$ and 6 g instant ocean sea salt in 250 ml distilled water. Pour into a carboy filled with 20 L distilled water and agitate.
2. Prepare 1 L 1-phenyl-2-thiourea (PTU) stock solution (20x). Dissolve 0.6 g PTU powder in 1 L distilled water. Add 1 ml of stock solution per 20 ml embryo medium to a final concentration of 0.003% as a working solution.
   CAUTION: PTU may cause systemic toxicity following inhalation or dermal exposure. Prepare and use in accordance with appropriate handling guidelines.
3. Prepare 1 L ethyl 3-aminobenzoate methanesulfonate (Tricaine) stock solution (20x). Dissolve 4 g Tricaine powder in distilled water. Adjust pH to 7 by adding 1 M Tris buffer (pH 9), and bring the final volume to 1 L with distilled water. Aliquot into 50 ml and store at -20 °C. Thaw before use.
   CAUTION: Tricaine can induce a loss of sensation. Prepare and use in accordance with appropriate handling guidelines.
4. Prepare 100 ml larval metronidazole (MTZ) working solution. Make fresh 15 mM MTZ solution by dissolving 0.25 g MTZ powder in 0.1% dimethyl sulfoxide (DMSO) in 100 ml embryo medium. Dissolve MTZ powder completely by vigorous shaking. Make fresh MTZ solution and use aluminum foil to prevent photocatalytic degradation of MTZ.
   CAUTION: MTZ may cause irritation. Prepare and use in accordance with appropriate handling guidelines.
5. Prepare 100 ml larval ethanol/metronidazole (EtOH/MTZ) working solution. Make fresh MTZ solution and use aluminum foil to prevent photocatalytic degradation of MTZ. Add 1.5 ml 100% ethanol into 100 ml MTZ solution to a final concentration of 1.5% just before use.
6. Prepare 500 ml adult MTZ working solution. Make fresh 10 mM MTZ solution by dissolving 0.83 g MTZ powder in 0.1% DMSO in 500 ml system water. Dissolve MTZ powder completely by vigorous shaking. Make fresh MTZ solution and use aluminum foil to prevent photocatalytic degradation of MTZ.
7. Prepare 1 L PEM buffer. Dissolve 30.2 g PIPES, 0.74 g EGTA, and 0.12 g $MgSO_4$ in distilled water. Adjust pH to 7 with NaOH. Bring final volume to 1 L with distilled water.

2. Preparation of Larval Zebrafish

1. Set up mating of [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}]^{15,17} adult zebrafish with *Tg(hand2:EGFP)*^{pd24} [13] adult zebrafish in mating tanks. Use dividers to conduct timed mating.
2. Remove the divider the following morning and allow fish to mate without disruption. Harvest embryos 2 hr later by straining system water and transferring the embryos into 100 mm Petri dishes with embryo medium.
3. Keep no more than 100 embryos per Petri dish. Under a stereomicroscope, remove unfertilized eggs using a glass pipette. Grow embryos at 28 °C and add phenylthiourea (PTU) to a final concentration of 0.003% after 10 hours-post-fertilization (hpf) to inhibit pigment development.

3. Ethanol, Metronidazole Treatment and Liver Regeneration in Larval Zebrafish

1. Prepare fresh EtOH solution by adding ethanol into embryo medium to a final concentration of 1.5%. Do not add PTU.
2. Treat embryos with 20 ml ethanol solution per Petri dish from 56 to 80 hpf. Cover Petri dishes with plastic wrap to prevent ethanol evaporation.
3. Sort out [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}*; Tg(hand2:EGFP)*^{pd24}] larvae with similar liver size, as determined by CFP expression, at 80 hpf. Do not use Tricaine when sorting the EtOH-treated larvae because this will cause high mortality rate with subsequent EtOH/MTZ treatment. Keep sorted larvae at a density of 30 embryos per Petri dish.
4. Prepare fresh 15 mM MTZ in embryo medium in 0.1% DMSO. Add proper amount of EtOH into MTZ solution to make a final concentration of 1.5%. Incubate larvae in 20 ml EtOH/MTZ solution per Petri dish for 24 hr at 28 °C. Cover Petri dishes with plastic wrap to maintain EtOH concentration and with aluminum foil to protect from light.
5. At the end of the treatment, remove EtOH/MTZ solution by transferring larvae to new Petri dishes with fresh embryo medium. Wash larvae 3 times with embryo medium and keep them in 20 ml embryo medium without PTU.
6. To sort out the larvae with near-complete ablation of hepatocytes, observe fluorescence under an epifluorescence microscope with the CFP filter at a magnification of 80X. Successful ablation results in minimal CFP fluorescence in the liver and significantly reduced liver volume.
7. To avoid death of the EtOH/MTZ-treated larvae, do not use Tricaine for sorting. Fix the larvae for immunostaining (refer to section 5) or keep sorted larvae in Petri dishes at 28 °C for further analyses.

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4. Chemical Screens in EtOH/MTZ-treated Larval Zebrafish

1. Bring the 96-well plates containing 10 mM chemical stocks in DMSO (refer to the Materials List for details regarding the commercial chemical libraries) from the -80 °C freezer. Cover with aluminum foil to prevent photocatalytic degradation of chemicals. Thaw the stock solutions at RT with gentle rocking.
2. Prepare chemical solutions by diluting 10 mM stock solutions with embryo medium to a final concentration of 50 µM (in 96 well plates: initial screen; in 1.5 ml tubes: retesting). Control larvae were treated with equal concentrations of DMSO in embryo medium.
3. Transfer larvae with near-complete hepatocyte ablation, which were treated with 1.5% EtOH/15 mM MTZ and sorted as aforementioned, to either 96-well (initial screen) or 24-well (retesting) plates prefilled with embryo medium at a density of 5 larvae per well. Use duplicate wells for each chemical.
4. Remove embryo medium and add either 250 µl (96-well plates) or 500 µl (24-well plates) chemical solutions to each well. Cover plates with aluminum foil and treat larvae for 50 hr at 28 °C. Inspect chemical-soaking larvae daily and remove any dead larvae from individual wells.
5. At the end of the chemical treatment, observe the number and/or intensity of CFP-expressing hepatocytes under an epifluorescence microscope with the CFP filter at a magnification of 80X. Photograph live larvae showing enhanced or reduced number and/or intensity of CFP-expressing hepatocytes with a digital camera for records.
6. Preserve larvae by formaldehyde fixation for confocal imaging as described in section 5.
7. To retest the chemicals that facilitate hepatocyte regeneration in the initial screen, examine their potency at higher and lower concentrations to find the most efficacious concentration with the procedures described in 4.1-4.5 ('retesting').

5. Larval Zebrafish Fixation, Immunostaining, and Confocal Imaging

1. Anesthetize larvae by adding Tricaine to a final concentration of 0.02%. Transfer 10-15 larvae to a 1.5 ml tube and wash once with phosphate-buffered saline (PBS). Remove PBS and add 1 ml of freshly prepared 2% formaldehyde in PEM buffer to fix O/N at 4 °C. CAUTION: Formaldehyde causes irritation and is known to be a human carcinogen. Handle in a chemical fume hood.
2. Discard fixing solution properly and then wash 3 times with PBS at RT. Fixed larvae can be kept in PBS at 4 °C for up to several days. Proceeding to the next step promptly gives better immunostaining results.
3. Carefully remove yolk and skin covering the liver area using a #55 forceps under a stereomicroscope.
4. Permeabilize and block larvae in Block buffer (4% bovine serum albumin and 0.3% Triton X-100 in PBS) O/N at 4 °C.
5. Incubate with rabbit anti-Collagen I antibody at a dilution of 1:100 in Block buffer O/N at 4 °C. Wash 5 times for 15 min each with Wash buffer (0.3% Triton X-100 in PBS).
6. Incubate with AlexaFluor 647 conjugated donkey anti-rabbit antibody at a dilution of 1:200 in Block buffer O/N at 4 °C. Wash 5 times for 15 min each with Wash buffer. Then wash once with PBS.
7. Gently transfer larvae to glass slides using a glass pipette, and orient fixed larvae with the left lateral side facing up. Remove excess PBS using Kimwipes. Add a drop of mounting media, and seal cover glass with nail polish. Conducting confocal imaging immediately is greatly recommended.
8. Use a confocal system equipped with a 40X/1.3 oil lens to capture images. Use laser strength at 20-50%. Capture Z stack images at 1 µm intervals.

6. Preparation of Adult Zebrafish

1. Set up mating of [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}]^{15,17} adult zebrafish in mating tanks. Harvest embryos the next morning by straining system water and transferring the embryos into 100 mm Petri dishes with embryo medium.
2. Keep no more than 100 embryos per Petri dish. Under a stereomicroscope, remove unfertilized eggs using a glass pipette. Grow embryos at 28 °C and add PTU to a final concentration of 0.003% after 10 hpf to inhibit pigment development.
3. At 4 dpf, use Tricaine to anesthetize larvae and sort out [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}] larvae. Wash out Tricaine by changing to fresh embryo medium several times. Allow larvae to recover at 28 °C until they achieve normal heart rate of 120-180 beats per minute[18].
4. Raise sorted larvae to 6-12 months old based on the standard procedure[19].

7. Ethanol, Metronidazole Treatment, and Liver Regeneration in Adult Zebrafish

1. Transfer 6-12 months old [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}] adult zebrafish to mating tanks using a net. Keep fish at a density of no more than 10 fish per tank.
2. Prepare fresh EtOH solution by adding EtOH in system water to a final concentration of 1%. Treat adult fish with 500 ml EtOH solution in each tank and maintain them in a 28 °C incubator.
3. Transfer adult fish to fresh EtOH solution daily, discard dead fish properly as a biohazard. Continuously treat animals for 72 hr before MTZ treatment.
4. Prepare fresh 10 mM MTZ solution in system water in 0.1% DMSO. Pre-warm the MTZ solution in a 28 °C incubator. Treat fish with 500 ml MTZ solution in 1 L crossing tank for 8 hr at 28 °C, protect from light using aluminum foil.
5. Observe hourly during the treatment and remove dead fish immediately. Discard dead fish properly as a biohazard. At the end of the MTZ treatment, wash out MTZ by transferring animals to fresh system water twice.
6. Allow animals to recover and maintain them in an incubator at 28 °C. Feed and transfer fish to fresh system water daily until the time point for analyses.

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8. Adult Zebrafish Liver Fixation, Immunostaining, and Confocal Imaging

1. Anesthetize adult fish with 0.02% Tricaine and euthanize in ice water for 15 min. Pat the fish dry on a paper towel and place it on a dissecting mat.
2. Expose gastrointestinal organs by removing skin and muscles as previously described[20]. Use scissors to cut the skin and underlying muscle along the belly from the anal fin to the operculum, and then posteriorly along the side of the fish back to the anal fin.
3. Put the fish in a 15 ml conical tube and wash once with PBS. Remove PBS and add 4 ml of freshly prepared 2% formaldehyde in PEM buffer to fix O/N at 4 °C.
4. Discard fixing solution properly, and wash 3 times with PBS at RT. Fixed samples can be kept in PBS at 4 °C for several days. Proceeding to the next step immediately gives better immunostaining results.
5. Dissect the whole gut with liver from the body cavity of the fish by cutting the esophagus. Embed the gastrointestinal organs with 4% low-melting agarose in a sectioning mold.
6. Use a vibratome to cut samples into transverse sections at 50 µm intervals in ice cold PBS, starting from the anterior end. Transfer sections to 6-well plate with PBS using a #1 paint brush.
7. Permeabilize and block sections in Block buffer O/N at 4 °C.
8. Incubate with rabbit anti-Collagen I antibody at a dilution of 1:100 in Block buffer O/N at 4 °C. Wash 5 times, 15 min each with Wash buffer.
9. Incubate with AlexaFluor 647 conjugated donkey anti-rabbit antibody at a dilution of 1:200 in Block buffer O/N at 4 °C. Wash 5 times, 15 min each with Wash buffer and once with PBS.
10. Gently transfer sections to glass slides using a #1 paint brush. Remove excess PBS using Kimwipes, add a drop of mounting media, and seal cover glass with nail polish. Conducting confocal imaging immediately is highly suggested.
11. Use a confocal system equipped with a 40X/1.3 oil lens to capture images. Use laser strength at 20-50%. Capture Z stack images at 1 µm intervals.

Representative Results

Figure 1 shows the development of an EtOH-induced fibrotic liver model in larval zebrafish. To optimize a protocol for exposing zebrafish larvae to EtOH, we first assessed EtOH toxicity. 2.5 days-post-fertilization (dpf) larvae were exposed to EtOH concentration 1%, 1.5%, or 2% for 24 hr followed by a concurrent 24 hr EtOH/MTZ treatment. Exposure to 2% EtOH caused high mortality, while nearly all larvae exposed to 1% EtOH or less showed minimal fibrogenic changes with rare deposition of extracellular matrix proteins like collagen. Based on these results, the larvae were pretreated with 1.5% EtOH for 24 hr (2.5-3.5 dpf) and then simultaneously treated with MTZ for 24 hours (3.5-4.5 dpf) (**Figure 1A**). The EtOH/MTZ-treated larvae showed morphological abnormalities including upward curvature of the trunk and tail, pericardial edema, and failure to inflate swim bladder. (**Figure 1B**). We used three transgenic lines to analyze fibrogenic changes in the EtOH/MTZ-treated larval livers: 1) *Tg(fabp10a:CFP-NTR)*^{gt1} line expresses the NTR gene fused with cyan fluorescence protein under the hepatocyte-specific *fabp10a* promoter[15]; 2) *Tg(Tp1:mCherry)*^{jh11} line marks cells with active Notch signaling (Notch-responsive cells, NRCs) or biliary epithelial cells (BECs) with nuclear mCherry[17], which expression is under the control of the TP1 module containing multiple RBP-Jk-binding sites; and 3) *Tg(hand2:EGFP)*^{pd24} line, which labels hepatic stellate cells (HSCs)[13]. DMSO-treated control [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}; *Tg(hand2:EGFP)*^{pd24}] livers showed no fibrillar type I collagen deposition[4,6,9] (**Figure 1C, C'**), whereas EtOH/MTZ-treated livers displayed elevated type I collagen accumulation at 25 and 50 hr-post-ablation (hpa) (**Figure 1D, D', E, E'**). Additionally, compared to DMSO-treated control livers (**Figure 1C"**), HSCs increased in number with the altered morphology from a star-like configuration to a myofibroblast-like shape with lost cytoplasmic processes in the EtOH/MTZ-treated regenerating livers (**Figure 1D", E"**). We observed two discrete populations of mCherry-expressing NRCs at 25 hpa in the EtOH/MTZ-treated regenerating livers: dim red NRCs (**Figure 1D"', inset, white arrows**) and bright red NRCs (**Figure 1D"', inset, yellow arrowheads**). At 50 hpa, hepatocyte-specific CFP began to co-express in NRCs with dim mCherry expression throughout the regenerating livers (**Figure 1E"', inset, white arrows**). These CFP and dim mCherry-coexpressing NRCs are evidently distinguishable from bright red NRCs that are negative for CFP (**Figure 1E"', inset, yellow arrowheads**). Previous studies showed that when hepatocyte proliferation was suppressed after partial hepatectomy (PHx), HPCs expanded with concomitant proliferation of HSCs[21]. Additionally, the HPCs and BECs shared histochemical markers[22]. Hence, our results indicate that the CFP and dim mCherry co-expressing NRCs portray a zebrafish-counterpart of HPCs that differentiate into hepatocytes by encountering Notch downregulation. The NRCs maintaining higher levels of Notch signaling may remain as cholangiocytes, which are differentiated BECs[23].

Figure 2 shows the development of an EtOH-induced fibrotic liver model in adult zebrafish. First, we exposed adult zebrafish to 1%, 1.5%, or 2% EtOH to assess viability. Most adult zebrafish did not survive more than 72 hr of exposure to EtOH concentrations greater than 1% (unpublished data). Therefore, for adult zebrafish, we pretreated the fish with 1% EtOH for 72 hr and followed with MTZ treatment (**Figure 2A**). By performing time-course analyses, we showed significantly elevated type I collagen deposition in the EtOH/MTZ-treated regenerating livers at 2, 3, and 4 days-post-ablation (dpa) (**Figure 2C', D', E'**) compared to DMSO-treated livers (**Figure 2B'**). Similar to that observed in the larvae, the CFP and dim mCherry co-expressing cells were detected in the EtOH/MTZ-treated regenerating livers of 12-month-old [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}] adult fish at 3 and 4 dpa (**Figure 2D, E, insets, white arrows**). These data indicate that the HPCs, responsive to Notch signaling, maintain their capacity to regenerate as hepatocytes in the presence of fibrogenic insult in adult zebrafish.

Figure 3 shows the representative chemical screening results using our ethanol-induced fibrotic liver model in larval zebrafish. To pinpoint bioactive compounds that accelerate hepatocyte regeneration in the fibrotic liver, we performed chemical genetic screens. We screened a library of 75 small molecules with well-characterized biological and pharmaceutical activities (Stem Cell Signaling Compound Library, Selleckchem) and a library of 1,000 less-characterized small molecules (ActiProbe-1K Library, TimTec) using [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{jh11}] larvae. We ablated the hepatocytes in the presence of 1.5% EtOH/15 mM MTZ and then exposed the larvae to 50 µM of the compounds for 50 hr (**Figure 3A**). A number of Wnt agonists such as SB 415286 and CHIR-99021, inhibitors of glycogen synthase kinase-3, promoted hepatocyte regeneration (**Figure 3C, D**) compared to DMSO (**Figure 3B**). Furthermore, [4-(1H-1,2,3,4-tetraazol-5-yl)-1,2,5-oxadiazole-3-ylamine], abbreviated as HTOA, a novel Wnt pathway activator, enhanced hepatocyte regeneration in the fibrotic liver as indicated by bigger regenerated liver (**Figure 3E**). These data suggest that our sustained fibrotic model provides an invaluable tool to discover small molecules that enhance hepatocyte regeneration.

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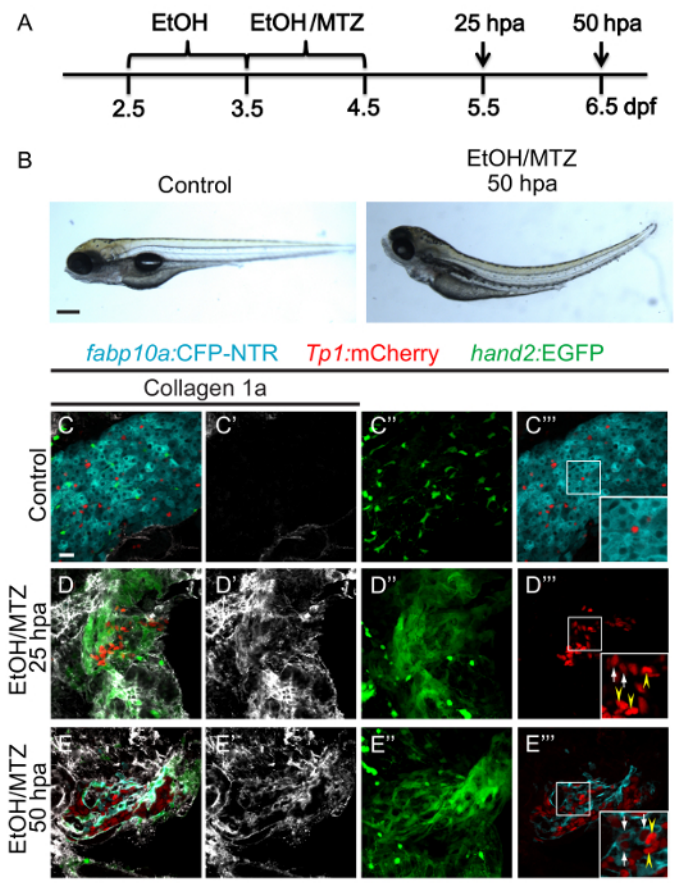

Figure 1. Development of an ethanol-induced fibrotic liver model in larval zebrafish. (**A**) Scheme illustrating the periods of EtOH and EtOH/MTZ treatment for establishing a fibrotic liver model in larvae. (**B**) At 50 hours-post-ablation (hpa), EtOH/MTZ-treated larval zebrafish developed morphological abnormalities including upward curvature of the trunk and tail, pericardial edema, and failure to inflate the swim bladder. (**C-C'''**) In the livers of [*Tg(fabp10a:CFP-NTR)*^{gt1}*; Tg(Tp1:mCherry)*^{ih11}; *Tg(hand2:EGFP)*^{pd24}] larvae treated with DMSO, ECM protein collagen 1a was almost undetectable (**C, C'**) and quiescent HSCs showed a star-like configuration (**C''**), whereas NRCs distributed among hepatocytes (**C, C''', inset**). (**D-E'''**) In the livers of EtOH/MTZ-treated larvae, elevated collagen 1a deposition was observed (**D, D', E, E'**). HSCs increased in number and lost complex cytoplasmic processes (**D'', E''**). At 25 hpa, mCherry-expressing NRCs are composed of two distinct populations. Yellow arrowheads indicate bright red NRCs (**D''', inset**), whereas white arrows indicate dim red NRCs (**D''', inset**). At 50 hpa, *Tg(fabp10a:*CFP-NTR) and *Tg(Tp1:*mCherry) co-expressing cells started emerging throughout the regenerating livers (**E''', inset, white arrows**), while the bright red NRCs had no CFP expression (**E''', inset, yellow arrowheads**). All confocal images are single plane images except **C'', D'', E'',** which are projection images. Scale bars: **B**, 100 µm; **C-E'''**, 20 µm. EtOH, ethanol; MTZ, metronidazole; hpa, hours-post-ablation; dpf, days-post-fertilization. Please click here to view a larger version of this figure.

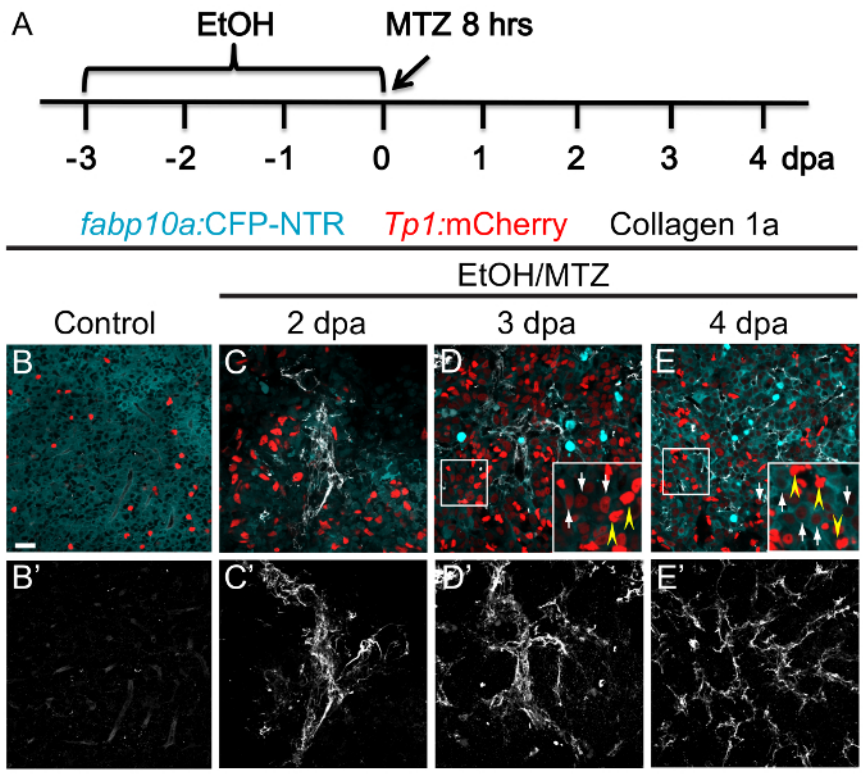

Figure 2. Development of an ethanol-induced fibrotic liver model in adult zebrafish. (**A**) Scheme illustrating the periods of EtOH and MTZ treatment for establishing a fibrotic liver model in adult. (**B-E'**) *Tg(fabp10a:*CFP-NTR), *Tg(Tp1:*mCherry), and collagen 1a expression in vibratome sections of DMSO- (**B** and **B'**) and EtOH/MTZ-treated (**C-E'**) adult zebrafish livers.(**B-B'**) In the DMSO-treated controls, collagen 1a was almost undetectable (**B'**) with no *Tg(fabp10a:*CFP-NTR) and *Tg(Tp1:*mCherry) co-expressing cells (**B**). (**C-E'**) In the EtOH/MTZ-treated regenerating livers, collagen 1a deposition was markedly elevated at 2, 3, and 4 dpa (**C', D', E'**) with a population of *Tg(fabp10a:*CFP-NTR) and *Tg(Tp1:*mCherry) co-expressing cells at 3 and 4 dpa (**D, E, insets, white arrows**). The bright red NRCs had no CFP expression (**D, E, insets, yellow arrowheads**). **B-E**, confocal single plane images. **B'-E'**, confocal projection images. Scale bar, 20 µm. EtOH, ethanol; MTZ, metronidazole; dpa, days-post-ablation. Please click here to view a larger version of this figure.

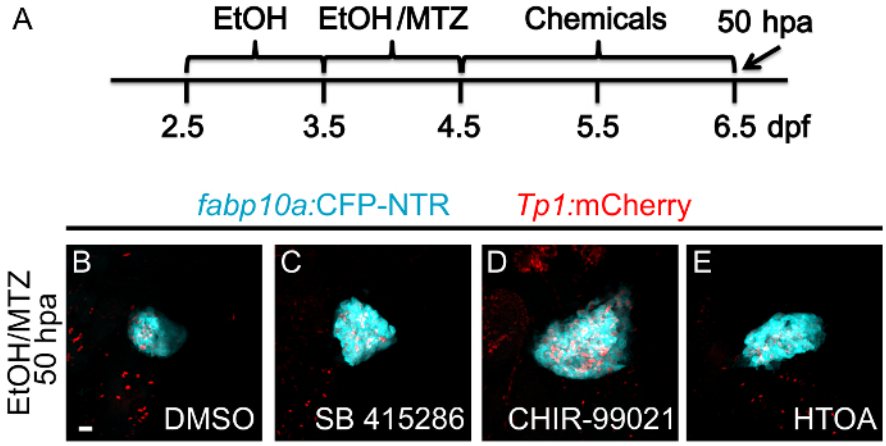

Figure 3. Representative chemical screening results using the ethanol-induced fibrotic liver model in larval zebrafish. (**A**) Scheme for the hepatocyte regeneration screen in the fibrotic liver. (**B-E**) Known and novel Wnt activators augment hepatocyte regeneration in the fibrotic liver. Confocal projections of the EtOH/MTZ-treated livers in the [*Tg(fabp10a:CFP-NTR)*^{gt1}; *Tg(Tp1:*mCherry^{jh11}] larvae that were administered with DMSO (**B**), SB415286 (**C**), CHIR-99021 (**D**), or a novel Wnt activator [4-(1H-1,2,3,4-tetraazol-5-yl)-1,2,5-oxadiazole-3-ylamine] (abbreviated as HTOA) (**E**). SB415286 and CHIR-99021 are glycogen synthase kinase-3 inhibitors. All images are confocal projection images. Scale bar, 20 µm. EtOH, ethanol; MTZ, metronidazole; hpa, hours-post-ablation; dpf, days-post-fertilization. Please click here to view a larger version of this figure.

Discussion

We observed HPC-mediated hepatocyte regeneration in the EtOH/MTZ-treated recovering livers, suggesting that even in the presence of substantial amount of ECM proteins including fibrillar type I collagen, the HPCs retain their competency to regenerate as hepatocytes. The MTZ only-treatment did not increase deposition of ECM proteins significantly, whereas the EtOH only-treatment did not induce HPC activation[15]. By utilizing the combined EtOH/MTZ treatment, we were able to investigate HPC-driven regeneration in the fibrotic liver. As near-complete elimination of hepatocyte elicits HPC-driven hepatocyte regeneration, it is critical to sort out MTZ-treated larvae based on the criteria of absence of hepatocyte-specific fluorescence and significantly reduced liver volume by clustered NRCs. In fish, the blood vessels of the gill and skin absorb the alcohol[24], so that there is no need to allow animals to drink *ad libitum* or require intragastric infusion as in mammalian models. We housed EtOH- and subsequent MTZ-treated adult fish in separate tanks without circulating the water. It is essential to transfer fish to fresh EtOH solution daily and keep the lid on to minimize the evaporation of EtOH. Although 48-72 hr EtOH/MTZ treatment efficiently induces ECM protein synthesis in our protocols, neither advanced fibrosis nor cirrhosis has been developed in these fish. Therefore, combination of MTZ with prolonged exposure of ethanol such as 12 weeks treatment[14], specifically in adult fish, may induce more severe liver damage to properly simulate and interrogate HPC-driven regeneration in chronic liver failure.

Zebrafish serves as an outstanding animal model for *in vivo* compound testing due to its small size, large progeny, transparency, and permeability to small molecules[25]. Using the fibrotic liver model in zebrafish, we identified a number of known and novel Wnt activators and Notch inhibitors that accelerated hepatocyte regeneration[15]. Our current fluorescence-based chemical screening protocol consists of several steps all performed manually including preparation of chemicals, transferring EtOH-treated/hepatocyte-ablated larvae to 24-well plates, treating chemicals, and analysis of effects of chemicals on hepatocyte regeneration. It specifically employs epifluorescence and/or confocal microscope to capture images for individual animal, which is laborious and time consuming. High-throughput platforms that automatically handle and acquire cellular-resolution imaging of zebrafish in combination with quantitative data collection will facilitate large-scale screening[26,27].These features will be strengthened if combined with automated drug dosing system that determines the range of chemical concentrations[28], which can give minimum toxicity with maximum efficacy for enhancing hepatocyte regeneration. Despite these limitations, as many critical players and main cell types of the liver are conserved between zebrafish and mammals[29], employing the *in vivo*-based small molecule screening using the zebrafish fibrotic liver model will catalyze valid discovery of novel therapeutic strategies for chronic liver disease.

Two additional groups independently have demonstrated that after near total loss of hepatocytes without fibrogenic insults, BECs transdifferentiated into hepatocyte through a step of dedifferentiation[30,31] with the assumption that cholangiocytes, fully differentiated BECs, primarily comprise NRCs/BECs in zebrafish. Although transdifferentiation can be one of the mechanisms of driving hepatocyte regeneration, our results indicate that the HPCs, which are known to constitute the majority of BECs in the zebrafish liver[32], downregulate Notch activity to differentiate into hepatocytes. Meanwhile, it is plausible to speculate that the fully differentiated BECs, cholangiocytes, maintain higher levels of Notch activity without converting to hepatocytes during regeneration. Intriguingly, in the presence of EtOH, as previously reported[13], we found that HSCs increased in number with the altered morphology from a star-like configuration to a myofibroblast-like shape with lost cytoplasmic processes. The activated HSCs characterized by synthesis of ECM proteins are the principle culprit responsible for abnormal wound healing during the integrated process of liver repair[4]. Although chronic injury often overrides the liver's remarkable capacity for regeneration, transplanted HPCs successfully repopulated the fibrotic/cirrhotic rat liver[33]. Hence, our zebrafish fibrotic liver model will be an essential tool for elucidating the molecular and cellular mechanisms that mediate the effects of sustained fibrosis on HPC-mediated hepatocyte regeneration. Furthermore, defining the multi-cellular crosstalk that balances regeneration and fibrosis by utilizing our zebrafish fibrotic liver model will further facilitate to design viable therapeutic strategies for chronic liver failure *in vivo*.

Disclosures

The authors declare that they have no competing financial interests.

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