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Using zebrafish to model liver diseases-Where do we stand?

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Abstract

Purpose of Review—The liver is the largest internal organ and performs both exocrine and endocrine function that is necessary for survival. Liver failure is among the leading causes of death and represents a major global health burden. Liver transplantation is the only effective treatment for end-stage liver diseases. Animal models advance our understanding of liver disease etiology and hold promise for the development of alternative therapies. Zebrafish has become an increasingly popular system for modeling liver diseases and complements the rodent models.

Recent Findings—The zebrafish liver contains main cell types that are found in mammalian liver and exhibits similar pathogenic responses to environmental insults and genetic mutations. Zebrafish have been used to model neonatal cholestasis, cholangiopathies, such as polycystic liver disease, alcoholic liver disease, and non-alcoholic fatty liver disease. It also provides a unique opportunity to study the plasticity of liver parenchymal cells during regeneration.

Summary—In this review, we summarize the recent work of building zebrafish models of liver diseases. We highlight how these studies have brought new knowledge of disease mechanisms. We also discuss the advantages and challenges of using zebrafish to model liver diseases.

Keywords

Cholestasis; Steatosis; Alcoholic liver disease; Non-alcoholic fatty liver disease; Fibrosis; Liver regeneration

Introduction

The liver is the largest gland in the body and plays vital roles in maintaining normal homeostasis. Metabolically, the liver is the prime site for detoxification, glycogen storage, and production of serum proteins, lipids, and hormone. It also generates bile that is critical for digestion and absorption of fat and vitamins in the small intestine. Whereas toxin exposure, viral infection, autoimmune responses, and genetic mutations can all serve as

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triggers, the resulting liver diseases often progress in a similar fashion [1]. Inflammation is observed first, followed by fibrosis as the liver forms scar tissue. Extensive scarring, distortion of the liver architecture, and loss of liver function are the characteristics of cirrhosis, which is the end stage of chronic liver diseases. Being constantly exposed to toxins, the liver has superior ability to repair itself. However, in cirrhosis, the extent of damage exceeds the self-regenerative capacity of the liver and liver failure occurs. Cirrhosis is among the leading causes of death worldwide [2], and the only available effective treatment is liver transplantation.

A better understanding of the mechanisms causing chronic liver diseases is key to improve diagnosis, treatment, and prevention. Although rodent models have been used extensively in liver research, it has become evident that no single animal model is capable of recapitulating every aspect of human liver diseases. Incorporating multiple animal models is more likely to yield complementary insights. The teleost zebrafish (*Danio rerio*) is a favorite vertebrate model organism in developmental biology. As the development and function of zebrafish organs are strikingly similar to those of humans, they have been successfully used to model hematopoietic disorders, cancer, cardiovascular diseases, muscle diseases, neurological disorders, kidney diseases, etc. [reviewed in 3]. In this review, we highlight the recent advances in establishing zebrafish models of liver diseases. We focus on cholestasis, alcoholic liver disease, non-alcoholic fatty liver disease, and liver regeneration. Zebrafish models of liver cancer have been recently reviewed elsewhere [4]. We demonstrate the technical advantages of using zebrafish to study liver biology. We also discuss the necessity to gain deeper knowledge of zebrafish liver homeostasis and physiology in order to define which aspects of different liver diseases can be modeled in fish.

Comparison of liver anatomy between zebrafish and mammals

The basic functional unit of mammalian liver is the liver lobule [5]. Each lobule has a hexagonal shape, composed of cords of hepatocytes that radiate from the central vein in the center of the lobule to the portal vein in the vertex. Hepatocytes can be divided into three different groups along the portocentral axis by their metabolic activities [6]. This so-called liver zonation is crucial for organ function. Hepatocytes secrete bile on the apical side through small channels known as bile canaliculi [5]. Bile fluid is then drained to the intrahepatic bile duct that is located within the portal triad along with portal vein and hepatic artery. On the basal side of hepatocytes are sinusoidal capillaries. Kupffer cells, the resident macrophages in the liver, also reside in these sinusoids. Vitamin A-storing hepatic stellate cells are located in the space of Disse that is between sinusoids and hepatocytes.

With the exception of Kupffer cells, all counterparts of major mammalian liver cell types have been reported in zebrafish (Table 1). At first glance, zebrafish liver anatomy seems very different from the mammals as the blood vessels are randomly distributed throughout the organ and there are no morphologically distinguishable central veins or portal veins [7]. Hepatocytes are arranged in tubules and the bile duct is located in the center. Metabolic zonation has not been described in the zebrafish liver. There are no portal triads. Instead, intrahepatic bile ducts and blood vessels are separated by hepatocytes (Fig. 1A,D). Histological analyses and transmission electron microscopy reveal the conservation and

distinction of zebrafish and mammalian liver anatomy. Similar to mammals, zebrafish hepatocytes secrete bile through bile canaliculi on the apical side. In mammals, bile canaliculi are located in between hepatocytes and are sealed by tight junctions to prevent leakage of bile [8]. In zebrafish, bile canaliculi are invaginations of the apical hepatocyte membrane that connect to biliary ductules (Fig. 1C,D). Bile canaliculi are also sealed by tight junctions, except that in zebrafish, tight junctions form between hepatocytes and biliary ductules. The blood vessels on the basal side of hepatocytes resemble the sinusoid capillaries in mammalian liver. Hepatic stellate cells also reside in between hepatocytes and endothelial cells and store lipid droplets (Fig. 1B). Lastly, whereas intrahepatic bile ducts in mammalian liver are often composed of multiple cholangiocytes with apparent epithelial morphology, most of the intrahepatic bile ducts in zebrafish liver are made up of one or two cholangiocytes (Fig. 1A,C). Only the cholangiocytes in the few large bile ducts that connect to the extrahepatic bile duct exhibit the typical columnar epithelial morphology [7, 9].

Liver development in zebrafish

The zebrafish liver arises from the foregut endoderm, similar to mammals. The first sign of hepatic specification is seen at around 22 hours post fertilization (hpf) when a group of anterior endodermal cells start to express hepatic transcription factors *hhx* and *prox1* [10]. Between 24 and 30 hpf, the gut undergoes left-ward bending, followed by budding of the liver primordium to the left side of the embryo [11]. The liver bud is very prominent at 48 hpf. Soon after, hepatoblasts differentiate into hepatocytes and intrahepatic biliary cells. Development of intrahepatic bile duct in mammals is through ductal plate [12]. At embryonic day 15.5 in mouse embryo, the hepatoblasts surrounding the branches of portal vein form ductal plate. Primary ductal plate structures exhibit an asymmetric composition with cells expressing cholangiocyte markers detected on the portal side and undifferentiated hepatoblasts located on the parenchymal side. Such asymmetry resolves as some of the hepatoblasts on the parenchymal side differentiate into cholangiocytes and join the periportal cholangiocytes to form a mature duct. In zebrafish, there is no evidence of ductal plate structures in the developing liver. Biliary cells first appear in vicinity of extrahepatic bile duct then expand to the rest of the liver [13]. Except for the large bile ducts that are associated with the extrahepatic bile duct, most intrahepatic bile ducts are formed when cellular processes derived from different biliary cells become interconnected. Concomitant with hepatic differentiation, endothelial cells and progenitors of hepatic stellate cells enter the liver [14, 15]. By 72 hpf, zebrafish liver contains a biliary ductal network and a vascular network with hepatocytes localized in between.

Tools to study liver pathophysiology in zebrafish

Zebrafish has emerged as an important model to study liver development and diseases in the past two decade. Its general, strengths include rapid external development, optical translucence, accessibility to genetic and chemical manipulation, ease of collecting hundreds of embryos on a weekly base, and relatively low-cost maintenance. Unlike the laboratory rodent strains, most zebrafish strains are not inbred and genetic background does not impose a significant impact on the phenotype. The zebrafish also offers features that are particularly useful for liver research: the molecular regulation of liver development is largely conserved

between zebrafish and mammals. The zebrafish liver becomes functional and develops all main cell types by 4 days post fertilization. Because zebrafish liver cells perform similar function as their mammalian counterparts, histological stains that are routine in mammalian liver research can be easily applied to zebrafish to examine liver pathophysiology (Table 1). Transgenic lines that express fluorescent proteins in hepatocytes, intrahepatic biliary cells, endothelial cells, and hepatic stellate cells are available, facilitating the observation of these cells both in live fish and in fixed livers (Table 1). These transgenic zebrafish also allow isolation of different liver cell types through fluorescence activated cell sorting for downstream transcriptome analyses [16]. Commercially available fluorescent lipid analogs, which are designed for visualizing lipid uptake and processing in live fish, have proven to be extremely sensitive in detecting hepatobiliary injuries (Table 1). For instance, the phosphoethanolamine analog PED6, when added to the water, is absorbed by the intestine and turns on fluorescence when cleaved by intestinal lipases [17]. When the function of the hepatobiliary system is intact, PED6 is processed through the liver and excreted into bile. It is transported through intrahepatic biliary network before becoming concentrated in the gallbladder. The fluorescent gallbladder signal can be easily detected using a low-power epifluorescent microscope. A reduction in PED6 uptake in the gallbladder is a strong indication of impaired hepatobiliary function. More recently, BODIPY-conjugated fatty acids with varying lengths of acyl chain have been used in conjunction with confocal microscopy to visualize the biliary network and the subcellular structures of the hepatocytes in live larvae [18]. BODIPY analogs have proven to be very effective in recognizing fat accumulation in steatosis and obstructions in the biliary tree [19, 20]. In this review, we provide a few examples of how the various tools described above can be applied in gain- and loss-of-function studies to test gene function in liver disease pathogenesis, and how they serve as straightforward readouts in forward genetic screens and chemical screens to identify novel regulators of liver development and diseases.

Zebrafish models of liver diseases

Neonatal cholestasis

Cholestatic jaundice affects 1 in every 2500 infants [21] and often indicates serious hepatobiliary and metabolic dysfunction. Cholestasis may result in significant morbidity and mortality due to pruritus, malnutrition, and complications arising from portal hypertension secondary to biliary fibrosis. Of the many conditions that cause neonatal cholestasis, the most common are biliary atresia (BA) (25–35%), genetic disorders (25%), metabolic diseases, and α 1-antitrypsin (A1AT) deficiency (10%) [22, 21]. We discuss the recent progress in using zebrafish to model neonatal cholestasis, with an emphasis on BA.

BA is a leading cause of neonatal cholestasis, and has an incidence of 1 in 10–19,000 in Europe and North America [23]. It is a multifaceted cholangiopathy that mainly destroys the extrahepatic bile duct and blocks bile flow. Even with Kasai portoenterostomy as a surgical intervention to improve biliary drainage, the liver disease progresses to end-stage cirrhosis in most children. BA accounts for nearly 40–50% of children who undergo liver transplantation. The etiology of BA is still unclear, but is likely caused by a combination of morphogenetic abnormalities, environmental factors, and inflammatory dysregulation [24].

The most well established animal model of BA is newborn Balb/C mouse infected with Rhesus rotavirus (RRV) [25–27]. Within a week of infection, these mice exhibit obstruction of the biliary tree and cholestasis that resemble many features of human BA. Whereas the RRV model has demonstrated the contribution of immune cells and inflammation to BA pathogenesis, recent studies using zebrafish have illustrated the involvement of genetic and epigenetic factors as well as environmental toxins in BA. Three groups independently performed genome-wide association studies (GWAS) and found single-nucleotide polymorphism (SNPs) or copy number variants (CNVs) that show strong association with BA susceptibility. They turned to the zebrafish model to validate the biological function of the candidate genes by using the morpholino oligonucleotide knockdown technique. Matthews' group revealed that knocking down *glypican 1* (*gpc1*), which encodes a cell-surface heparan sulfate proteoglycan, and *adducin 3* (*add3*), which encodes a membrane skeletal protein, both resulted in decreased PED6 uptake in the gallbladder and a less complex intrahepatic biliary network [28, 29]. *gpc1* knockdown also led to a smaller gallbladder [28]. In addition, Hedgehog signaling activity was increased in both *gpc1* and *add3* knockdown larvae. Injection of Sonic Hedgehog protein into the wild-type fish caused biliary abnormalities, whereas treatment with an Hedgehog inhibitor partially rescued the biliary phenotypes in *gpc1* and *add3* knockdown larvae. Excessive Hedgehog activation has also been observed in children with BA [30], suggesting that Hedgehog signaling has evolutionarily conserved roles in cholangiocyte development. The Sindhi group knocked down a different candidate gene, *ADP-ribosylation factor 6* (*arf6*), which encodes a GTP-binding protein [31]. They observed similar intrahepatic biliary defects to those seen in *gpc1* and *add3* knockdown larvae. They further linked the ARF6 and EGFR pathways in regulating intrahepatic biliary system development. These findings demonstrate that zebrafish can be used not only to validate GWAS discoveries, but also to identify molecular pathways that are altered by variants.

A recent study using zebrafish has provided strong evidence to support the toxin theory for BA pathogenesis. By conducting the fluorescent lipid analog assay in zebrafish, Lorent et al. screened for compounds in the extracts of *Dysphania* species that cause BA-like symptoms in herds of sheep and cows in New South Wales, Australia [32]. This approach led to the discovery of a novel isoflavonoid, biliaryresone, which specifically obstructs the extrahepatic bile duct in zebrafish larvae. In a subsequent study, the authors showed that genes involved in glutathione (GSH) metabolism were upregulated in biliaryresone-treated larval livers, and hepatic GSH was depleted upon biliaryresone treatment [33]. Taking advantage of the power of *in vivo* imaging in zebrafish, the endogenous GSH redox state was assessed by a redox-sensitive fluorescent biosensor, revealing that the extrahepatic biliary cells had a more oxidized GSH pool than the hepatocytes and intrahepatic biliary cells both with and without biliaryresone treatment, which may explain why the extrahepatic biliary cells are specifically targeted by the toxin. In support of the contribution of GSH to BA, inhibition of GSH synthesis sensitized both the extrahepatic and intrahepatic biliary cells to the toxin and increasing GSH levels by pharmacological approaches suppressed biliaryresone-induced injury. Importantly, the specific damage of biliary cells caused by biliaryresone treatment, as well as the link between GSH and biliaryresone also holds true in mouse [34, 32]. This study

provides an excellent example of using chemical screening approaches in zebrafish to identify novel disease mechanisms.

Studies of mutant zebrafish have indicated that DNA hypomethylation is a potential mechanism to initiate molecular cascades leading to BA. In the *ducttrip* mutant, a mutation in the gene for S-adenosyl homocysteine hydrolase, *ahcy*, results in global inhibition of DNA methylation [35]. In both *ducttrip* mutants and fish treated with an inhibitor of DNA methylation, the intrahepatic bile ducts are disorganized and sparse. Activation of the interferon- γ pathway has been observed in human BA and the mouse RRV model [36, 37]. In zebrafish, inhibition of DNA methylation increased the expression of interferon- γ pathway genes, and injection of interferon- γ into the zebrafish recapitulated the biliary phenotypes. Further demonstrating the relevance of the zebrafish findings to human BA, the authors showed that DNA methylation was greatly reduced in the cholangiocytes in BA patients compared to those in patients with other types of neonatal cholestasis. Cofer et al. conducted methylation microarray analysis in livers of BA patients and showed that Hedgehog pathway genes *SHH* and *GLI2*, as well as PDGF pathway gene *PDGFA* were hypomethylated in BA [38]. Interestingly, injection of PDGF-AA protein dimer induced biliary phenotype in zebrafish larvae and activation of Hedgehog pathway increased *PDGFA* expression, suggesting a potential link between Hedgehog pathway and PDGFA in BA.

Zebrafish have also been used to model inherited cholestatic diseases in children. In Alagille syndrome, mutations in the Notch ligand gene *JAGGED1* and receptor gene *NOTCH2* cause bile duct paucity [39]. Inhibition of Notch signaling activity in zebrafish also suppresses biliary differentiation and morphogenesis [7]. Knockdown of genes involved in arthrogryposis-renal dysfunction-cholestasis syndrome [40–42] and North American Indian Childhood Cirrhosis [43] leads to intrahepatic biliary defects in zebrafish. Progressive familial intrahepatic cholestasis (PFIC) is a group of inherited neonatal cholestatic disorders that primarily injure the hepatocytes [44]. PFIC is characterized by impaired bile secretion due to mutations in the genes encoding the canalicular transporters ATP8B1, ABCB11, and ABCB4, and tight junction protein TJP2. Zebrafish form canaliculi on the apical side of hepatocytes, and the ultrastructure of zebrafish canaliculi is very similar to the mammalian counterparts (Fig. 1). The zebrafish canalicular transporter proteins have high (>60%) homology with the human proteins [45]. The Abcb11 protein is detected in the canaliculus in zebrafish, similar to its localization in the mammalian liver [14]. It will be interesting to generate the canalicular transporter mutants and explore the feasibility of modeling PFIC in zebrafish.

Polycystic liver disease

Polycystic liver diseases (PCLD) are genetic disorders of the biliary epithelium, characterized by progressive formation of fluid-filled cysts throughout the liver parenchyma [46]. They result in massive enlargement of the liver, which compresses the neighboring organs and impair their function. The patients are also at high risk of developing cholangitis, fibrosis, cirrhosis, and even cancer. Mutations in the genes encoding cilia-associated proteins Polycystin1/PKD1, Polycystin2/PKD2, and PKHD1 are found in PCLD patients who also have polycystic kidney [47]. Mutations in *SEC63* and *PRKCSH* are responsible for isolated

PCLDs. Both genes encode proteins that are located in the endoplasmic reticulum (ER) and function in translocation of glycoproteins into the ER. The zZebrafish genome contains orthologs of the genes mutated in PCLD. The LaRusso group showed that knockdown of *sec63*, *prkcsb*, and *pkd1a* in zebrafish by antisense morpholino oligonucleotides caused the formation of hepatic cysts [48]. Similarly, both *prkcsb* knockdown and overexpression led to pronephric cysts, abnormal body curvature, and *situs inversus*, but not hepatic cysts [49]. Monk et al. identified a *sec63* zebrafish mutant carrying a missense mutation predicted to change a highly conserved amino acid. Interestingly, aside from ER stress and steatosis, the mutant liver does not develop hepatic cysts [50]. Several mechanisms have been shown to cause hepatic cysts in mammals, including ductal plate malformation, disrupted cholangiocyte polarity, increased fluid secretion from the cholangiocytes, as well as defects in primary cilia [51]. Intrahepatic bile duct development in zebrafish does not involve the formation of ductal plate. The intrahepatic biliary cells do not have a typical epithelial appearance with the exception of those composing the large bile ducts. Primary cilia and fluid secretion have not been well characterized in zebrafish biliary cells. The differences in bile duct development and cholangiocyte physiology may explain why perturbing PCLD-related gene function in zebrafish does not fully recapitulate the cystic phenotypes seen in human.

Alcoholic liver disease

Alcoholic liver disease (ALD) results from excessive alcohol intake and is one of the most common causes of liver-related morbidity and mortality [52]. ALD progresses from hepatic steatosis, steatohepatitis, fibrosis, cirrhosis, and eventually hepatocellular carcinoma [53]. Chronic ALD is an adult disease and takes years of alcohol abuse. Whether zebrafish can also be used to model chronic ALD has not been convincingly demonstrated. It has been shown that adult zebrafish develop steatosis, steatohepatitis and fibrosis when being continuously housed in 1% ethanol for a long period of time [54]. However, such treatment may prevent the animal from feeding properly, making it difficult to determine to what extent the liver injuries are induced directly by alcohol.

In recent years, binge drinking is becoming an alarming global health problem [55]. Binge alcohol exposure causes liver inflammation, steatosis, hepatocyte death, and promotes activation of hepatic stellate cells and fibrogenesis [56]. Thus it is crucial to identify therapeutic agents that target multiple aspects of the disease. Oral feeding of alcohol only induces hepatic steatosis in rodent models [57, 58], and development of inflammation and fibrosis requires a second insult [59, 60]. Zebrafish larvae are well suited to study the effects of acute alcohol exposure as alcohol can be added to the water and does not cause lethality [61]. Genes and pathways necessary for alcohol metabolism are highly conserved in zebrafish and they readily take up and metabolize alcohol at as early as 4 days post fertilization [62, 61, 63, 64]. Intriguingly, exposing 4-day-old larvae to 2% ethanol for just 24 hours is sufficient to induce steatosis, angiogenesis, recruitment of macrophages, and activation of hepatic stellate cells [61, 15, 16]. Using this model, the Sadler group showed that the sterol response element binding protein (Srebp) pathway [61] and activation of unfolded protein response (UPR) were required to induce hepatic steatosis in acute ALD liver injury [65, 66]. Blocking ethanol metabolizing enzymes and oxidative stress alleviated

UPR activation and reduced ethanol-induced steatosis in the zebrafish larvae [67]. Activating transcription factor 6 (Atf6) [65], one of the three UPR sensors, was both necessary and sufficient to cause hepatic steatosis independent of Srebp activity. Our group, on the other hand, investigated the roles of vascular endothelial growth factor (VEGF) signaling in acute alcoholic liver injury [16]. We showed that blocking VEGF receptor Kdr1 activity by either a pharmacological inhibitor or genetic mutation suppressed hepatic steatosis, angiogenesis and fibrogenesis induced by acute ethanol treatment. To understand how these three aspects of ethanol-induced acute liver injury were linked to each other and the mechanism of action of VEGF signaling, we performed quantitative PCR on the FACS-sorted liver cells. We found that both hepatic stellate cells and endothelial cells, but not hepatic parenchymal cells, exhibited robust *kdr1* expression upon ethanol treatment, suggesting that angiogenesis and fibrogenesis are directly targeted by Kdr1 inhibition. In *cloche* mutants that do not form hepatic endothelial cells, acute ethanol treatment still caused steatosis and fibrogenesis and both could be blocked by Kdr1 inhibition. We propose that in acute ALD, steatosis and fibrogenesis can be uncoupled from angiogenesis. VEGF signaling regulates activation of hepatic stellate cells, which in turn maintains steatosis in hepatocytes. Although anti-VEGF agents seem to have beneficial effects on multiple aspects of acute ALD, our data showed that blockade of VEGF signaling did not prevent fibrogenesis in the presence of ethanol. In this regard, our zebrafish study has provided insightful assessment for the use of anti-VEGF agents in acute ALD treatment: they may promote liver recovery from alcohol-induced injury upon cessation of alcohol consumption, but may not be useful for individuals with an active alcohol addiction.

Non-alcoholic fatty liver disease (NAFLD)

NAFLD is characterized by excessive fat accumulation in the liver that is not related to alcohol overconsumption [68]. It also has a broad spectrum of disorders, including steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. NAFLD is closely associated with insulin resistance, fatty diet, drug-induced liver injury, and metabolic syndromes. Due to the increasing prevalence of obesity and type II diabetes, NAFLD has become the fastest rising cause of chronic liver disease worldwide [69]. Similar to human, zebrafish develop hepatic steatosis in response to environmental triggers such as hepatotoxin, fasting, and diet containing high content of fat, cholesterol, or fructose (summarized in Table 2). Through reverse genetics, researchers have generated mutants and transgenic zebrafish to manipulate the function of known genes and investigate the molecular mechanisms underlying hepatic steatosis. The results of these studies have been reviewed by others [19] and are listed in Table 2. Forward genetic screen in zebrafish identified several mutants with hepatic steatosis. *foie gras (foigr)* mutant was the first genetic zebrafish model of fatty liver disease that was isolated in a screen for hepatomegaly [42]. The *foigr* gene is the zebrafish ortholog of human *trafficking protein particle complex 11/ TRAPPC11* that is involved in ER-to-Golgi trafficking [70]. Loss of TRAPPC11 function causes UPR responses and ER stress contributing to steatosis [42, 66, 71]. In other liver morphology-based screens, Thakur et al. reported a mutant of *CDP-diacylglycerol-inositol 3-phosphatidyltransferase* and connected phosphatidylinositol synthesis with ER stress and steatosis [72]. Nussbaum et al. identified a *guanosine monophosphate synthetase* mutant in which reduced production of reactive oxygen species resulted in hepatic steatosis [73].

Several groups have incorporated oil-red-o staining into the genetic screens to effectively detect steatosis. The *redmoon* mutant carries a mutation in the *slc16a6a* gene that encodes a ketone body β -hydroxybutyrate transporter [74]. Characterization of this mutant revealed a previously unrecognized role of hepatic ketone body transport in fasting-induced steatosis. A recent large-scale forward screen isolated 19 novel mutants displaying hepatomegaly and/or steatosis during post-developmental stages [75]. It is anticipated that identification of the underlying mutations and the affected molecular pathways will advance our understanding of NAFLD etiology.

Liver regeneration

Because specific anti-fibrotic therapies are not yet available, whole organ transplantation remains as the only effective treatment for end-stage liver diseases. Given the overwhelming epidemiology of chronic liver diseases, there is an urgent need for alternative therapeutic strategies. Hepatocyte replacement is a logical option, but hepatocytes do not propagate well *in vitro*. Obtaining large amount of hepatocytes is very costly and difficult because of the limited availability of donor livers. The growing new knowledge on stem cell biology has provided new avenue of cell therapy for liver diseases. Now it is possible to reprogram differentiated cells to a pluripotent state and convert them into hepatocytes and even mini-liver buds in culture [76, 77]. Studying liver regeneration in animal models has crucial implications for improving the current methodology of differentiating and propagating hepatocyte *in vitro*.

Liver has exceptional proliferative capacity that is often sufficient to repair itself after acute liver injury. Two-thirds partial hepatectomy (PH) in rodents is one of the most commonly used animal models for studying liver regeneration after acute injury [78, 79]. In PH, the liver regenerates through replication of existing hepatocytes. One-third PH has been performed in adult zebrafish [80]. The liver regains its original volume by 7 days after the surgery via compensatory growth of the remnant hepatocytes [81]. Analyses of genetic mutants and transgenic zebrafish have revealed important mediators of hepatocyte proliferation in PH, including the cell cycle regulator Uhrf1 [80], BMP, FGF [81], and Wnt signaling [82].

In massive or chronic liver injury where the proliferative capability of hepatocytes is severely compromised, facultative hepatic progenitor cells/stem cells may be activated to replenish the lost hepatocytes [83]. It has been proposed that hepatic progenitor cells/stem cells belong to the biliary lineage based on their ductal-like morphology and gene expression. By using an adenoassociated virus (AAV) vector that expresses Cre recombinase and loops out floxed sequences in all hepatocytes, several groups conducted hepatocyte lineage-tracing experiments in classic adult mouse liver injury models, including those that are thought to activate hepatic progenitor cells/stem cells [84–86]. In every model they tested, preexisting hepatocytes were the main source of regenerating hepatocytes. One plausible explanation is that despite the severity of hepatocellular damage in these models, sufficient number of hepatocytes have survived to regenerate the organ. In zebrafish, the nitroreductase-mediated cell ablation system driven by a hepatocyte-specific promoter is capable of eliminating nearly all hepatocytes [87]. Under this condition, Notch-active

intrahepatic biliary cells have been shown to convert into hepatocytes to regenerate the liver [88–90]. Biliary-to-hepatocyte conversion is blocked in the mutants with bile duct paucity and liver regeneration is severely perturbed in these animals [89]. Choi et al. showed that the Wnt ligand Wnt2bb was required for the proliferation of biliary-derived new hepatocytes [88]. Luo group, on the other hand, demonstrated that the bile duct transcription factor Sox9b became activated upon hepatocyte ablation and was essential for biliary-to-hepatocyte conversion [89]. They also showed that inhibiting Notch signaling blocked Sox9b activation and biliary-to-hepatocyte conversion. Huang et al. extended the analyses on Notch signaling and revealed that the biliary cells experienced different levels of Notch signaling upon hepatocyte ablation. Low levels of Notch promoted the conversion whereas high levels of Notch inhibited the conversion [90]. It is noteworthy that most of the hepatocyte ablation studies were conducted at the larval stage when the liver was still rapidly developing. Some Notch-active cells might still be hepatoblasts rather than differentiated biliary cells. An increase of Notch-active cell number was seen in the adult zebrafish after hepatocyte ablation and some of them also turned on the hepatocyte marker expression [88, 90]. In mouse hepatocytes can enter an intermediate state in liver injury and express both biliary and hepatocyte markers [91]. It will be important to perform lineage-tracing experiments in adult zebrafish after hepatocyte ablation to distinguish whether the Notch-active cells also expressing hepatocyte markers are the biliary cells that undergo dedifferentiation to give rise to hepatocytes, or they are the surviving hepatocytes that become Notch-active in response to injury.

The ease of ablating hepatocytes and tracking liver regeneration in live zebrafish larvae have inspired researchers to conduct chemical screens to identify regulators of liver regeneration. Huang et al. found that liver regeneration was augmented by Wnt agonists and inhibited by Wnt antagonists and described a negative feedback loop between Notch and canonical Wnt signaling in hepatocyte regeneration. Ko et al. identified bromodomain and extraterminal domain (BET) inhibitors in the screen and showed that they blocked biliary-to-hepatocyte conversion when added during hepatocyte ablation [92]. When BET inhibitors were added after ablation, they suppressed the proliferation of newly formed hepatocytes and delayed their maturation. In human chronic liver diseases, the number of hepatic progenitor cells known as oval cells is correlated with disease severity [93, 94]. However, these cells have poor ability to regenerate liver. In this regard, compounds that promote biliary-to-hepatocyte conversion in zebrafish may have the potential to promote transdifferentiation of hepatic progenitor cells/biliary cells to hepatocytes in humans.

Conclusion and Future Perspectives

Zebrafish has been increasingly recognized as a valuable animal model for studying liver diseases and is complementary to the cell culture and rodent model systems. Further characterization of the zebrafish liver disease models will bring new insights into the molecular and cellular mechanisms underlying disease pathogenesis. Conducting high throughput forward genetic screens and chemical screens in zebrafish offers tremendous opportunity for identifying new therapeutic targets and treatment.

There are still many questions that need to be addressed in order to ensure the proper use of zebrafish in modeling liver diseases. Most zebrafish liver studies focus on the larval stage mainly for the following reasons: 1) zebrafish liver is already functional and contains all cell types at the larval stage. 2) 4- to 5-day old larva remains largely transparent, making it easy to observe the liver *in vivo*. 3) At this stage, zebrafish obtains nutrients almost exclusively from yolk. 4) Morpholino oligonucleotides can still effectively knockdown gene function during the larval stage. However, the larval phenotypes likely only mimic what occurs during early stages of liver diseases, but offer limited information on disease progression. There may not be enough time for fibrosis to develop in the larvae. The adaptive immune system is not mature at the larval stage [95], thus the hepatobiliary injury does not induce similar immune responses in zebrafish larvae as in patients. Therefore, it will be necessary to follow these models beyond the larval stage and investigate what aspects of the liver damage seen in patients can be recapitulated in zebrafish. Along the same line, although it is clear that environmental insults and genetic mutations can induce steatosis and hepatic stellate cell activation in zebrafish, neither advanced fibrosis nor cirrhosis has been reported. It could be due to the fact that not enough adult studies have been conducted. Differences in hepatocyte and cholangiocyte physiology could also be important contributing factors, but our knowledge on this topic is very limited. Lastly, measuring serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) is the gold standard for evaluating liver function in patients and rodent models, and have been used in a few studies [96, 97]. It is important to further develop these assays in zebrafish so that one can more closely compare the liver phenotypes between species.

In this era of genomic analyses, GWAS has become a routine methodology to uncover genetic loci that are associated with disease phenotypes. The main challenge for the follow-up studies of patient GWAS is to determine which variants are indeed causative. Four groups have used zebrafish to validate GWAS variants in BA patients, as well as in patients with elevated liver enzyme levels [28, 98, 31, 29]. Furthermore, CRISPR/Cas9-mediated targeted mutagenesis can now be applied in zebrafish not only to generate null mutations, but also to precisely recapitulate the same mutations found in patients. It is also possible to mutagenize multiple targets in the same injected fish, allowing rapid testing of multiple candidate genes from GWAS [99]. When generating zebrafish mutants to validate GWAS variants, it is important to examine the liver phenotypes in both larva and adult. A deeper understanding of the zebrafish immune system and liver physiology is also needed to characterize the liver phenotypes that are related to immune responses and liver metabolism, respectively. Nevertheless, zebrafish represent an animal model that is an ideal combination of speed of testing, cost effectiveness, and homology to human. It is highly feasible to establish a pipeline for discoveries of candidate variants from patients, direct testing for biological relevance in zebrafish, and validate the findings in patients' livers.

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Fig. 1.

Anatomy of zebrafish liver. (A–C) Transmission electron microscopy images of the liver from a 5-month old wildtype adult zebrafish. (A) Low magnification image showing a sinusoidal capillary (S) that is located in the center of the image, and on the basal side of the hepatocytes (Hep). The black square marks an intrahepatic biliary ductule (BD) on the apical side of a hepatocyte. (B) showing a lipid-storing hepatic stellate cell (HSC) that is located in between the hepatocyte (Hep) and sinusoidals (S). (C) is a high magnification view of the black square in (A). The bile canaliculus (Can) is invagination of the hepatocyte apical membrane and contains tightly packed actin-rich microvilli. The bile canaliculus directly associates with the biliary ductule (BD). Tight junctions (arrowheads) form between the hepatocyte and biliary ductule to seal the bile canaliculus. (D) Diagram showing the basic architecture of zebrafish liver. (E–H) Confocal images of the liver in the hepatocyte reporter line *Tg(fabp10a:dsRed)* (E), the intrahepatic biliary cell reporter line *Tg(EPV.Tp1-Mmu.Hbb:EGFP)* (F), the endothelial cell reporter line *Tg(kdr1:Hsa.HRAS-mCherry)* (G), and the hepatic stellate cell reporter line *TgBAC(hand2:EGFP)* (H) at 5 days post fertilization (dpf). (E–H) Ventral views, anterior is to the top.

Table 1

Tools to study zebrafish liver biology

A. Transgenic lines that label different liver cell types.		
Transgenic line	Cell type labeled	Reference
<i>Tg(fabp10a:dsred)</i>	Hepatocytes	[1]
<i>Tg(EPV.TPI-Mmu.Hbb:EGFP)</i>	Intrahepatic biliary cells	[2, 3]
<i>Tg(krt18:EGFP)</i>	Intrahepatic biliary cells, extrahepatic biliary cells, gallbladder	[4]
<i>Tg(kdrl:Hsa.HRAS-mCherry)</i>	Endothelial cells	[5]
<i>TgBAC(hand2:EGFP)</i>	Hepatic stellate cells	[6]
<i>Tg(wt1b:EGFP)</i>	Hepatic stellate cells	[7, 6]
B. Antibodies that label different liver cell types or subcellular structures.		
Antibody	Cell type/structure labeled	Reference
Prox1	Hepatoblasts, hepatocytes, intrahepatic biliary cells	[8]
Hnf4a	Hepatocytes	[8]
Alcam/Activated leukocyte cell adhesion molecule	Intrahepatic biliary network	[9]
2F11/Annexin A4	Intrahepatic biliary network	[10, 8]
Cytokeratin18	Intrahepatic biliary cells including the terminal ductules	[11]
Abcb11/BSEP	Bile canaliculus	[12, 9]
Mdr1	Bile canaliculus	[2, 13]
Desmin	Hepatic stellate cells	[6, 14]
GFAP/Glial fibrillary acidic protein	Hepatic stellate cells	[15, 6]
C. Histological stains.		
Histology	Purpose	Reference
Hematoxylin and eosin	Overview of liver histology	[16]
Mason's Trichrome	Type 1 collagen/Fibrosis	[17]
Oil red o	Neutral triglycerides and lipids/Steatosis	[18]
Nile red	Neutral lipid droplets/Steatosis	[19]
Periodic Acid-Schiff stain	Glycogen deposition	[20]
D. Assays to examine liver function.		
Assay	Purpose	Reference
PED6	Lipid uptake and transport	[21, 22]
BODIPY-conjugated fatty acids	Lipid metabolism and transport	[23]

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Table 2**Zebrafish models of non-alcoholic fatty liver disease**

A. Chemical treatment.			
Chemical	Phenotype and Mechanism		Reference
Fructose	ER stress, mitochondrial dilation and activation of mTOR pathway.		[1]
Tunicamycin, Thapsigargin, Brefeldin A	Activation of unfolded protein response (UPR) pathways.		[2]
γ-hexachloro-cyclohexane	Oxidative stress leading to ER stress and mitochondrial malfunction.		[3, 4]
Thioacetamide (TAA)	Increased apoptosis, lipogenesis and lipid peroxidation.		[5, 6]
Perfluorooctane sulfonate (PFOS)	Inhibition of β-oxidation coupled with activation of the nuclear hormone receptors peroxisome proliferator-activated receptor alpha (PPARα) and constitutive androstane receptor/pregnane X receptor (CAR/PXR).		[7, 8]
Tributyltin (TBT)	Impaired triglyceride accumulation and transcriptional regulation of lipid metabolism.		[9]
Amiodarone	Altered lipid homeostasis.		[10]
Bisphenol A (BPA)	Upregulation of the endocannabinoid system that induces steatosis.		[11]
B. Dietary treatment.			
Diet	Mechanism		Reference
High fat diet High fat plus high cholesterol diet	Obesity-related steatosis		[12–16]
C. Genetic mutants.			
Mutant	Gene Function	Liver phenotype	Reference
foie gras/trappc11	ER-to-Golgi trafficking	Hepatic steatosis; impaired hepatic function.	[17–19]
ducttrip/ahcy	Methionine metabolism	Hepatic steatosis; mitochondrial dysfunction; liver degeneration.	[20]
cdipt	Phospholipid synthesis	Hepatic steatosis due to ER stress and defects in <i>de novo</i> phosphatidyl synthesis.	[21]
sec63	ER-associated protein involved in translocation of glycoproteins	ER stress and hepatic steatosis.	[22]
gmgs	Guanosine monophosphate synthetase	Steatosis due to down-regulated activity of the small GTPase Rac1 and ROS production.	[23]
stk11	Serine/Threonine kinase 11 regulating phosphorylation of the nutritional sensor AMP-kinase	Fasting-induced hepatic steatosis; glycol depletion.	[24]
red moon/slc16a6a	β-hydroxybutyrate transporter required for hepatocyte secretion of ketone bodies during fasting.	Increased neutral lipids and induction of hepatic lipid biosynthetic genes when fasted.	[25]
slc7a3a	Arginine transporter required for arginine-dependent nitric oxide synthesis.	Fasting-induced steatosis.	[26]
mbtps1	Membrane-bound transcription factor peptidase site 1	The mutant is protected from chronic ER stress-induced steatosis.	[27]
cnr1 cnr2	Cannabinoid receptor	Adult <i>cnr2</i> mutants are susceptible to hepatic steatosis.	[28]

A. Chemical treatment.

Chemical	Phenotype and Mechanism	Reference
<i>socs1a</i>	Suppressor of cytokine signaling-1a and show impaired methionine metabolism. <i>cnr1</i> mutants are protected from steatosis. Hepatic steatosis and insulin resistance linked to elevated Jak-STAT5 signaling	[29]

D. Transgenic models.

Transgenic line	Transgene expressed	Liver phenotype	Mechanism	Reference
<i>Tg(-2.8fabp10a:HBV.HBx- GFP)</i>	Hepatitis B virus <i>X</i> protein (<i>HBx</i>)	Hepatic steatosis, hepatitis, liver hypoplasia.	Increased lipogenesis, fatty acid synthesis, lobular inflammation.	[30]
<i>Tg(fabp10a:GFP-gank)</i>	<i>ankyrin repeat protein (gankyrin)</i>	Hepatic steatosis, increased hepatocyte apoptosis and liver failure.	Alteration of lipid metabolism, increased expression of microRNAs.	[31]
<i>Tg(fabp10a:dnfgr1- EGFP)</i>	<i>dominant-negative fibroblast growth factor receptor 1 (dnfgr1)</i>	Hepatic steatosis, cholestasis, ballooning degeneration of hepatocytes.	Perturbation of lipid and bile metabolism	[32]
<i>Tg(fabp10a:EGFP-YY1)</i>	ubiquitous transcription factor <i>yin yang 1 (yy1)</i>	Hepatic steatosis.	Accumulation of hepatic triglycerides (TGs) by inhibiting CHOP-10 expression	[33]
<i>Tg(fabp10a:Tetoff- CB1R:2A:eGFP)</i>	<i>cannabinoid receptor 1 (cb1r)</i>	Hepatic steatosis.	Stimulates Srebp-1c, Acc1 and Fas expression.	[34]
<i>Tg(actb2:EGFP-nrlh3)</i> <i>Tg(fabp2:EGFP-nrlh3)</i>	Global (<i>actb2</i> promoter) or intestinal (<i>fabp2</i> promoter) expression of <i>Liver X</i> receptor (<i>nrlh3</i>)	Global overexpression of <i>nrlh3</i> induces steatosis; Intestine-specific overexpression suppresses steatosis caused by high-fat diet.	Overexpression of <i>nrlh3</i> in the intestine affects transport of lipids to the liver.	[35]

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