

Liver development in zebrafish (*Danio rerio*)

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Abstract

Liver is one of the largest internal organs in the body and its importance for metabolism, detoxification and homeostasis has been well established. In this review, we summarized recent progresses in studying liver initiation and development during embryogenesis using zebrafish as a model system. We mainly focused on topics related to the specification of hepatoblasts from endoderm, the formation and growth of liver bud, the differentiation of hepatocytes and bile duct cells from hepatoblasts, and finally the role of mesodermal signals in controlling liver development in zebrafish.

Keywords: zebrafish; model organism; liver development; endoderm

Introduction

Liver is an essential organ in the body and performs a number of vital activities including metabolism, detoxification and homeostasis. Hepatocytes make up the majority of a liver and exert most of the liver's functions, such as metabolism of a wide range of endogenous and exogenous substances, synthesis of blood protein and clotting factors, storage of glycogen, amino acids, fat, and iron, as well as secretion of bile (Blouin et al., 1977; Kawamoto et al., 1996; Pack et al., 1996; Field et al., 2003). Liver diseases including hepatitis B, cirrhosis and hepatocellular carcinoma (HCC) are worldwide health problems and cause high death toll annually, making great threaten to human health and social development (Dienstag, 2008). Therefore, it is important to illustrate liver development and its cellular and molecular mechanism. The knowledge learned will not only generate new approaches for early monitoring, prevention and therapy of the liver diseases but also help to guide *in vitro* culture and transplantation of the liver. In

this review, we briefly summarized the major progresses in zebrafish (*Danio rerio*) in the following aspects of liver development: the origination and specification of hepatoblasts within endoderm, the formation and growth of liver bud, the differentiation of hepatocytes and biliary cells from hepatoblasts and the roles of mesodermal signals during the liver development.

Zebrafish as an excellent model system for studying liver development

Zebrafish belongs to teleosts whose liver has distinguishable histological characteristics compared with that in the mammalian. Its portal veins, hepatic arteries and large biliary ducts are distributed stochastically within the hepatic parenchyma but are not grouped in portal tracts as in the mammalian. Besides, hepatocytes are arranged as tubules that enclose small bile ducts rather than as bilayered hepatocyte plates appeared in the mammalian. The intrahepatic bile ducts are derived from the bile canaliculi and form a network of biliary channels; the bile is then col-

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lected in the gallbladder through large ducts and an extrahepatic biliary system (Pack et al., 1996; Lorent et al., 2004).

In mammals, liver organogenesis begins with the establishment of a population of hepatic precursor cells within the ventral foregut endoderm, followed by the specification of definitive hepatoblasts. The hepatoblasts delaminate from the epithelial layer to form a discrete liver bud, and undergo rapid proliferation to increase the size of the bud. Finally, the hepatoblasts differentiate into functional hepatocytes and biliary duct cells (Zaret, 2002; Duncan, 2003). Although the process of liver initiation and development at the anatomic level is well established, its molecular and cellular mechanisms are relatively understudied through genetic approaches, and only limited information has been obtained up to now. The study of liver development is mainly constrained by the model animals used such as mouse and chicken, which cannot be used for large-scale genetic analysis. Zebrafish, on the other hand, has become a popular vertebrate model during last decade because both genetic and experimental embryological methods can be easily applied to this animal (Grunwald and Eisen, 2002). Zebrafish is a small tropical freshwater fish native to South Asia and is a common aquarium fish around the world. In 1981, Streisinger and his colleagues published a landmark paper describing the genetic procedures for producing clones of homozygous diploid zebrafish (Streisinger et al., 1981). At the Cold Spring Conference in 1994, zebrafish was formally established as a model animal for the vertebrate development study (Concordet and Ingham, 1994; Kahn, 1994; Mullins et al., 1994; Solnicakrezel et al., 1994). In 1996, an entire issue of *'Development'* journal reported two independent large-scale mutagenesis screens for phenotypic mutants and the analysis of some of the obtained mutants in zebrafish (Driever et al., 1996; Haffter et al., 1996). With more and more exciting findings published in a wide range of journals, zebrafish has shown its great potential in life science. Now it has become an ideal model organism for the study of vertebrate development and disease, functional genomics, organ function, behavior, toxicology and drug discovery. This is mainly attributed to some of its advantages, including its easy and economical maintenance, short generation time, external fertilization, large numbers of rapidly developing embryos produced per mating, and the external development of transparent embryos (Streisinger et al., 1981; Grunwald and Eisen, 2002). Given that fundamental developmental programs are well shared among vertebrate animals, the studies on zebrafish

should naturally contribute to a better understanding of the molecular and genetic mechanisms of development underlying other classes of vertebrates including human.

In addition to the above mentioned characteristics, zebrafish has some unique advantages for studying liver development. In mammals, the embryonic liver is an early hematopoietic organ, therefore, mutations affecting liver or blood development often cause anemia and even early lethality during embryogenesis that will complicate the study of liver development in the mammalian system (Reimold et al., 2000). Furthermore, mammalian embryogenesis occurs intrauterinely which makes the embryonic liver inaccessible for the study of the processes of liver development *via* a direct genetic approach. On the other hand, the blood (primitive) in zebrafish forms in the ICM (intermediate cell mass) first and subsequently (definitive) in the PBI (posterior blood land) and lastly kidney, not in the liver (Detrich et al., 1995; Thisse and Zon, 2002; Jin et al., 2009). Meanwhile, since zebrafish obtains its nutrient mainly from yolk during embryogenesis, zebrafish can survive and go on to develop relatively normally for a few days without the cardiovascular system (Stainier, 2001). These advantages allow the studies on liver development and disease in zebrafish even the mutations affecting blood development. Last but not least, the availability of a number of transgenic fish lines harboring the reporter genes *gfp* and/or *rfp* driven by a variety of endoderm-specific promoters or the heat-shock promoter not only allow researchers to follow the developmental processes of the liver *in vivo* but also greatly facilitate mutant screening based on the expression of the reporter genes (Table 1).

The origination and specification of hepatoblast

The endoderm gives rise to the hepatoblast. Fate mapping experiments have revealed that, at early gastrula stage (6 hpf, hours post-fertilization), endoderm cells located in the ventral part tend to differentiate to liver bud while endoderm cells situated on the dorsal side are apt to give rise to pancreas. These liver-only progenitors are located considerably further from the dorsal organizer than the pancreas-only progenitors. Clones that contribute to both liver and pancreas are prone to lie in more intermediate positions (Ward et al., 2007).

Currently, there are two different hypotheses regarding the initiation of liver progenitor cells in zebrafish. Based on the fact that the liver specific marker *ceruloplasmin* (*cp*) can be detected in the dorsal endoderm at 16 hpf which is

Table 1
Transgenic reporter fish used in the studies of liver development in zebrafish

Transgenic line	Tissue expression	References
<i>Tg(gutGFP)^{S584}</i>	Express the <i>gfp</i> gene in the entire alimentary canal and endodermal component of all accessory organs	Field et al., 2003
<i>Tg(sox17:GFP)^{S870};</i> <i>Tg(sox17:DsRed)^{S903}</i>	Express the <i>gfp</i> and <i>rfp</i> genes in the <i>sox17</i> positive endoderm cells, respectively	Chung and Stainier, 2008
<i>Tg(lfabp:EGFP);</i> <i>Tg(lfabp:dsRed)</i>	Express the <i>gfp</i> and <i>rfp</i> genes in the liver, respectively	Her et al., 2003; Shin et al., 2007
<i>Tg(hsp70:dnBmpr-GFP)^{w30}</i>	Express a dominant negative form of the <i>Xenopus</i> Bmp receptor type 1a fused to GFP after heat shock	Pyati et al., 2005, 2006
<i>Tg(hsp70:noggin3)</i>	Express Noggin3 after heat shock	Chocron et al., 2007
<i>Tg(hsp70:bmp2b)^{fl3}</i>	Express Bmp2b after heat shock	Chocron et al., 2007
<i>Tg(hsp70:alk8)</i>	Express alk8 (a type I Bmp receptor) after heat shock	Shin et al., 2007
<i>Tg(hsp70:dnfgfr1-EGFP)</i>	Express a dominant negative form of Fgfr1 fused to GFP after heat shock	Lee et al., 2005b
<i>Tg(ptf1a:GFP)^{jh1}</i>	Express GFP in the developing ventral pancreas	Godinho et al., 2005; Chung et al., 2008

prior to the liver morphogenesis, Korzh et al. (2001) hypothesized that liver progenitors might be differentiated before the initiation of alimentary canal morphogenesis and the liver bud was formed later by migration and aggregation of these liver progenitor cells. Similar hypothesis is also applied to the pancreas initiation because the pancreas marker *pdx1* is first detected at the 10-somite stage (~14 hpf) which is way ahead of the pancreas morphogenesis (Biemar et al., 2001; Wallace and Pack, 2003). However, so far there is no concrete evidence to demonstrate that the early *cp* positive cells do contribute to the formation of liver bud at a later stage. As for the *pdx1* positive cells, it is likely that they mainly contribute to the endocrine pancreatic cells but not to the exocrine pancreas (Field et al., 2003). The second hypothesis is based on the fact that the expression of two transcription factors *hhx* and *prox1*, two key hepatoblast markers, are detectable in the endoderm region that later gives rise to liver bud, suggesting that liver progenitor cells are differentiated from the endoderm cells to form *in situ* liver bud after the formation of endoderm rod. The latter hypothesis is supported by data from anatomic studies (Field et al., 2003; Wallace and Pack, 2003) and by studies on mutants related to liver development (Ober et al., 2006; Shin et al., 2007; Huang et al., 2008).

Liver bud formation and growth

Liver is an accessory organ of the foregut. Based on the results observed in the GutGFP transgenic zebrafish *Tg(gutGFP)^{S584}* (expressing the *gfp* reporter gene in the

entire alimentary canal and endodermal component of all accessory organs), Field et al. (2003) proposed that the liver morphogenesis process can be arbitrarily divided into two phases: budding and growth. The budding phase occurs from 24 to 50 hpf and can be further divided into three stages. At the budding stage I, endoderm cells caudal to the pharyngeal region aggregate to form endoderm rod at 24 hpf. At 28 hpf, the endoderm rod segment under the first somite starts to thicken, which marks the beginning of liver morphogenesis. From 28 hpf (budding stage II), the anterior thickening region, which refers to liver primordium, increases in size and bends to the left side (a process termed as gut-looping) with respect to the middle line (Horne-Badovinac et al., 2003), and then covers the outer curvature of intestinal bulb by 30 hpf. The formation of a furrow between the liver bud and the adjacent esophagus marks the beginning of last phase of liver budding at approximately 34 hpf. Along with the expansion of the furrow, the hepatic duct is shaped to connect liver and intestine at the end of budding stage III. The liver now appears to locate between the duct of Cuvier, anteriorly, and the mid-level of fin bud, posteriorly (Field et al., 2003) (Fig. 1).

At the subsequent growth stage, the liver undergoes dramatic changes in its size, shape and placement because of rapid cell proliferation. Hepatoblasts also start to differentiate into functional hepatocytes and bile duct cells. By 96 hpf, the liver extends across the midline ventral to esophagus to form the second liver lobe (right lobe). It touches the pericardial cavity anteriorly and overlaps with the anterior portion of residual yolk (Field et al., 2003) (Fig. 1).

Cellular and molecular control of liver development in zebrafish

In merely less than a decade, the power of using zebrafish

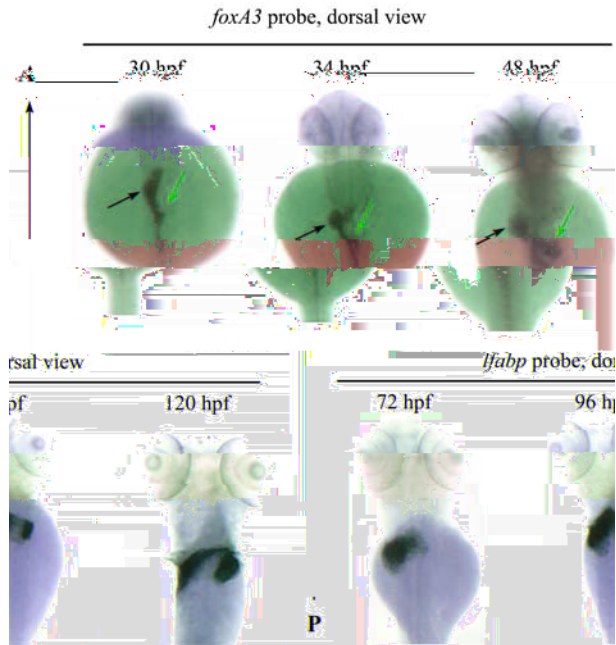


Fig. 1. Dorsal view of liver development in zebrafish. The *foxA3* probe was used in whole-mount *in situ* hybridization (WISH) for embryos at 30, 34 and 48 hpf as shown (black arrow: liver; red arrow: pancreas). The *lfabp* probe was used in WISH for embryos at 72, 96 and 120 hpf. A, anterior; P, posterior.

to study liver development has been demonstrated by a number of high-quality publications. In the following, we summarized the main achievements of these studies (Fig. 2).

Signaling molecules (*Bmp*, *Fgf*, *Wnt*, *Shh* and *RA*)

Early in 1975, Douarin found that hepatocytes differentiated from endoderm required interactions with adjacent precardiac mesoderm in chick (Douarin, 1975), and the same result was confirmed in mouse (Gualdi et al., 1996). It was ascribed to the signaling molecules such as fibroblast growth factors (Fgfs) from the cardiac mesoderm (CM) (Jung et al., 1999) and bone morphogenetic proteins (Bmps) from the septum transversum mesenchyme (STM) (Rossi et al., 2001). During the budding stage in zebrafish, liver cells are observed adjacent to lateral plate mesoderm (LPM) cells at the dorsal face and to yolk ball at the ventral face directly (Horne-Badovinac et al., 2003). Therefore, the question arises whether zebrafish LPM cells express signaling molecules functioning to regulate liver development similar to Fgf and Bmp signaling in mouse and chick. The

type. Therefore, as found in mouse (Zhao et al., 2005), Gata4 and Gata6 have redundant function during early stage of liver development and are both essential for liver growth in zebrafish (Holtzinger and Evans, 2005).

Retinol binding protein 4 (rbp4) is not only expressed in the adult zebrafish liver, but also detected in the yolk syncytial layer (YSL). *rbp4* is negatively mediated by Nodal and Hedgehog (Hh) signaling, but positively regulated by RA signal. It regulates liver budding and may be also required for migration of liver primordia. *rbp4* morphants show two liver buds which may be due to improper migration of liver primordia. Since *rbp4* also regulates the activity of *fibronectin 1 (fn1)*, *fn1* may act on liver primordia migration directly (Li et al., 2007). Knockdown of *vegfc* in zebrafish embryos also results in multiple liver and pancreatic buds; however, the expressions of both anterior liver bud and posterior pancreatic bud markers are unaffected, indicating that the anteroposterior patterning and organ differentiation of the gut endoderm is normal (Ober et al., 2004). Studies on the zebrafish *cloche (clo)* mutant show that endothelial cells do not appear to be essential for liver budding (Field et al., 2003), which is different from what have been observed in mouse (Matsumoto et al., 2001).

Def (digestive-organ-expansion-factor), a novel nuclear-localized protein, is required for digestive organ expansion but not organ initiation and cell differentiation. Loss-of-function of *def* selectively up-regulates the expression of a newly identified p53 isoform $\Delta 113p53$ (human counterpart $\Delta 133p53$) that leads to up-regulation of cell-cycle-related genes but not apoptotic-related genes to arrest the expansion growth of major digestive organs, including liver (Chen et al., 2005, 2009). Two other factors, *nil per os (npo)* encoding an RNA-binding protein and *leg1 (liver-enriched gene 1)*, have also been shown to play a crucial role in the expansion growth of embryonic liver in zebrafish (Mayer and Fishman, 2003; Cheng et al., 2006).

Pescadillo (Pes) is highly conserved from yeast to human and can be detected firstly at 48 hpf in the liver primordium, followed by the reduced RNA level at 72 hpf. During normal development between 72 hpf and 144 hpf, the liver extends over the yolk surface and this process is associated with rapid consumption of the yolk. On the other hand, in the *pes* mutant (generated *via* retroviral vector insertional mutagenesis method) the liver expansion is arrested beginning at 72 hpf, the mutant yolk is not consumed and the mutant fish dies at 144 hpf (Allende et al., 1996).

Expression of the *ubiquitin-like protein containing PHD*

and ring finger domains-1 (uhrfl) gene is enriched in the liver bud and fully developed liver but is rare in the adult zebrafish liver. Analysis of the zebrafish *hi272* line, which bears an insertion in *uhrfl*, reveals that *uhrfl* regulates liver apoptosis and proliferation but has no visual effects on hepatocytes histological manifestation. In the *uhrfl* mutant, the liver is shaped as a ball whereas the right form should be crescent-shaped in wild type at 120 hpf. In addition, *uhrfl* also functions in zebrafish liver regeneration (Sadler et al., 2007).

Some other factors have also been identified to play roles in the liver development. Rai et al. found that DNA methyltransferase 2 (Dnmt2) (Rai et al., 2007) but not Dnmt1 (Rai et al., 2006) was required for liver development in zebrafish. Dnmt2 is a maternal protein and its specific expression in liver is only detectable at 72 hpf. Since the expression of late but not early liver differentiation markers are impaired in the *dnmt2* morphants, Dnmt2 may affect the late stage of hepatocyte differentiation through cytoplasmic RNA methylation (Rai et al., 2007). Ftz-f1 belongs to the nuclear receptor type of transcription factor and is expressed in different forms. The IIA form of Ftz-f1 is the major transcript during zebrafish early liver morphogenesis, indicating its potential role in liver development (Lin et al., 2000). Although no direct evidence is obtained up to now, the function of Ftz-f1 homologs in human (Li et al., 1998) and rat (Galarneau et al., 1996) has indicated this notion. HNF-1 is a vertebrate transcription factor that contains a divergent homeodomain. In the zebrafish *hnf1* mutant (*vhnf1^{hi548}* allele), liver is poorly formed with undifferentiated hepatocytes at 72 hpf (Sun and Hopkins, 2001). The liver marker gene *hhex*, which is also essential for liver formation (Wallace et al., 2001), expresses at a low level in the *vhnf1^{hi548}* mutant, indicating its role downstream of vHnf1. It is proposed that vHnf1 controls liver development through regulating the balance of the *pdx1-shh* network (Sun and Hopkins, 2001). Recently, Cheng et al. (2006) reported their analysis of promoter regions of 51 liver-enriched genes by searching putative binding sites for Hnf1, Hnf3, Hnf4 and Hnf6, and revealed that these four liver-enriched transcription factors form a network to control the expression of liver-specific or liver-enriched genes in the liver. Interestingly, chromatin remodeling factors histone deacetylase 1 (Hdac1) (Noel et al., 2008) and Hdac3 (Farooq et al., 2008) have also been shown to play important but relatively distinct roles during liver specification and development in zebrafish. It seems that Hdac3 acts through inhibiting its unique target growth differentiation factor 11 (Gdf11), a member of the

transforming growth factor beta family, to promote liver development (Farooq et al., 2008).

Genes essential for differentiation of hepatocytes and bile duct cells

Onecut transcription factors (Oc) play important roles during zebrafish liver and pancreas development. *onecut1* (*oc1*, mammalian ortholog *hmf6*) and its downstream gene *vhnf1* are both essential for the biliary system development. Knockdown or overexpression of either gene perturbs the development of intrahepatic but not extrahepatic bile ducts (Matthews et al., 2004). As the third member of *onecut* gene family and also a functional ortholog of mammalian *hmf6*, *onecut3* (*oc3*) regulates the earliest stages of zebrafish biliary development. Expression of *oc3* is obviously reduced in *oc1*-deficient embryos, and *vice versa*. Therefore, members of the Onecut family seem to interact with each other to regulate biliary development (Matthews et al., 2004).

In addition, *jagged 1, 2, 3* and *notch 1a, notch 1b, notch 2*, and *notch 5* are each expressed in liver at 48 hpf and 72 hpf, exactly the time when bile ducts form. Absence of *jagged* or *notch* genes affects a series of zebrafish tissues including biliary system development, causing a phenotype comparable with the human Alagille Syndrome (AGS) (Lorent et al., 2004). Disruption of the biliary and pancreatic ductular system also appeared in *hhex* morphant (Wallace et al., 2001).

Genes essential for hepatopancreatic duct development

During morphogenesis of liver and exocrine pancreas, liver bud and exocrine pancreatic bud are linked by the hepatopancreatic duct. However, the nature and function of the hepatopancreatic duct have been studied only recently. In the zebrafish *fgf10* mutant, the hepatopancreatic duct epithelium becomes malformation and the contiguous cells fate is disordered (Dong et al., 2007). Based on the detailed molecular and cellular study, it is established that Fgf10 secreted from the adjacent mesenchyme functions not only to refine the boundaries between the hepatopancreatic duct and organs, but also to prevent the differentiation of the proximal hepatic and pancreatic cells into each other (Dong et al., 2007). Interestingly, in addition to its role in liver organogenesis, a distinct role of histone deacetylase 1 (Hdac1) in extrahepatopancreatic duct morphogenesis has also been identified (Noel et al., 2008).

Zebrafish as a model for liver disease study

In addition to its contribution to the study of liver development, zebrafish is increasingly being used to study liver diseases. Lam et al. (2006) generated liver tumors in zebrafish by treating the fish with carcinogens and then obtained the gene set for liver tumors *via* microarray hybridization. Next, they cross-compared the zebrafish liver tumor gene set with the gene sets obtained from four cancer types (liver, gastric, prostate and lung) in human. Astonishingly, they found that the zebrafish liver tumor gene set intersects most with the gene set from human liver tumors and this lays the foundation to establish zebrafish as a model system to study liver tumorigenesis (Lam et al., 2006). The fact that α -hexachlorocyclohexane, thioacetamide and alcohol each can induce hepatic steatosis in zebrafish successfully convinces the scientific community that zebrafish is a good model system to study steatohepatitis (Braunbeck et al., 1990; Amali et al., 2006; Passeri et al., 2009). More importantly, zebrafish serves as a genetic model system for studying the molecular mechanism behind hepatic steatosis. For example, it has been shown that mutation in a novel gene *foie gras* leads to hepatomegaly likely due to enlarged lipid-filled hepatocytes in the mutant liver. Detailed study of the *gonzo* mutant (mutated in the *membrane-bound transcription factor protease 1* gene) showed that alcohol induced hepatic steatosis was largely through activation of the pathway mediated by the sterol response element binding protein (SREBP) transcription factors (Passeri et al., 2009). On the other hand, mutation in the *S-adenosylhomocysteine hydrolase* (*ahcy*) gene increases *tnfa* expression to cause the hepatic steatosis phenotype in the *ducttrip* (*dtp*) mutant (Matthews et al., 2009). In a screen for zebrafish hepatomegaly mutants, Sadler et al. (2005) found that mutations in a class C vacuolar sorting protein gene *vps18* and a tumor suppressor gene *nf2* both conferred hepatomegaly due to defects in the biliary system development.

Conclusion

In recent years, zebrafish has shown a full potential in the studies of vertebrate liver development. The combination of forward and reverse genetics studies, and classical genetics analysis coupled with direct embryos observation and manipulation, as well as its particular suitability for the study of liver organogenesis, will no doubt strengthen our understanding of the cellular and molecular mecha-

nism of liver development.

Liver formation is a complicated process. Thus, there must be multiple genes and signaling molecules involved in this process. Up to now, we have known that Fgf, Bmp, Wnt and RA signaling pathways are involved in the initiation and differentiation of hepatocyte in zebrafish, but the precise relationship among them remains unclear. Furthermore, only a handful of factors had been identified to affect liver budding and growth. For example, *hhex* acts to control the initiation and budding of liver progenitors. *prox1* is required for the outgrowth of liver bud and migration of hepatoblasts whilst members of *gata* family, as pan-endodermal factors, are crucial for liver development. However, these few factors and pathways are not sufficient for understanding the whole process of liver initiation and development. Therefore, while further studies of known genes and existing mutants will provide us with new information for the understanding of liver organogenesis, more liver-defective mutants need to be identified for illustrating mechanisms that are behind the mutant phenotype observed. The identification of such mutants would lead to revealing the genes that are crucial for liver organogenesis. Though the method for target gene knockout in zebrafish is not sophisticated yet at present, we believe breakthrough will be made in the near future. Apparently, knowledge gained from studying liver development will help to guide the fields such as liver transplantation, gene therapy and tissue engineering, and to benefit human being as a whole.

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