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Making it new again: Insight into liver development, regeneration, and disease from zebrafish research

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Abstract

The adult liver of most vertebrates is predominantly comprised of hepatocytes. However, these cells must work in concert with biliary, stellate, vascular and immune cells to accomplish the vast array of hepatic functions required for physiological homeostasis. Our understanding of liver development was accelerated as zebrafish emerged as an ideal vertebrate system to study embryogenesis. Through work in zebrafish and other models, it is now clear that the cells in the liver develop in a coordinated fashion during embryogenesis through a complex yet incompletely understood set of molecular guidelines. Zebrafish research has uncovered many key players that govern the acquisition of hepatic potential, cell fate and plasticity. Although rare, some hepatobiliary diseases – especially biliary atresia - are caused by developmental defects; we discuss how research using zebrafish to study liver development have informed our understanding of and approaches to liver disease. The liver can be injured in response to an array of stressors including viral, mechanical/surgical, toxin-induced, immune-mediated, or inborn defects in metabolism. The liver has thus evolved the capacity to efficiently repair and regenerate. We discuss the emerging field of using zebrafish to study liver regeneration and highlight recent advances where zebrafish genetics and imaging approaches have provided novel insights into how cell plasticity contributes to liver regeneration.

1. Introduction

The liver carries out a diverse array of functions that are essential to physiological homeostasis. Among these are the ability to synthesize serum proteins including clotting factors, lipoproteins, and proteins such as albumin that generate colloidal pressure of serum, urea detoxification, energy storage, metabolism of xenobiotics, including most drugs, toxins and alcohol, the ability to respond to insulin to maintain energy homeostasis and the production of bile which aids in digestion. All of these functions are conserved from mammals to fish and work in zebrafish on liver development and disease over the last decade

has complimented nearly a century of work in mammalian systems. These topics have recently been reviewed by experts in the field (Goessling and Sadler, 2015; Goessling and Stainier, 2016; Wilkins and Pack, 2013a).

The breadth of knowledge distilled from research across the vertebrate lineage has provided a rich understanding of how the liver forms, functions, regenerates and what processes can cause diseases in this organ. In recent years, there is an increased appreciation of how pathways important for hepatogenesis also contribute to liver regeneration and disease. Here, we describe work to uncover the basis of hepatobiliary development and regeneration in zebrafish and discuss how this work has provided insight into some diseases.

Loss of liver function is fatal and hepatic failure is a leading cause of death, accounting for 1.4% of all deaths in the U.S. annually (Murphy et al., 2012). Liver related mortality is even higher in regions of the world where viral hepatitis viral infection is poorly controlled or health care options are limited (Lozano et al., 2012). Mammals have a notoriously poor ability to regenerate just about every organ, except for the liver, which can mobilize either differentiated hepatocytes or progenitor cells to repeatedly repopulate the liver following injury. Despite this, the level of liver injury caused by toxin overload, metabolic imbalance, infection or other insults can overwhelm its regenerative capacity, leading to liver failure. Thus far, the only curative treatment for liver failure is liver transplantation, which is severely limited by the availability of liver donors (Martin et al., 2013). Devising alternatives to liver transplant or expanding the potential donor pool is a major goal of hepatic regenerative medicine. Promoting regeneration by reinitiating liver development, generating progenitor cells that can repopulate the damaged liver or boosting the growth of a small piece of transplanted liver are all areas that hold promise for achieving this goal. Progress will be expedited by research that provides a better understanding of the genes and pathways important for liver development and regeneration. In addition to the liver, the heart, fin, and spinal cord can also regenerate in zebrafish (reviewed by (Gemberling et al., 2013)), making research in this model useful for providing insight into the mechanisms that prevent effective regeneration in mammals. Here we focus on the discoveries in the field of liver development and regeneration using zebrafish that have provided novel insights into how liver disease occurs and strategies to defend against it.

2. Liver development: Zebrafish as a paradigm

As part of the digestive system, the liver is derived from endoderm that also gives rise to the other digestive organs (pancreas, gut, gall bladder). Hepatocytes derive from bi-potential hepatobiliary progenitor cells and recent studies in zebrafish and other systems indicate that cell plasticity is important both during development and in repopulating the adult liver after injury. Liver development has been studied extensively in zebrafish and this topic has recently been thoroughly reviewed (Goessling and Stainier, 2016; Wilkins and Pack, 2013a).

Compared to other organs discussed in chapters in this volume, the liver has a relatively simple structure. The hepatocyte comprises up to 60% of total cells of the liver – even higher in zebrafish - and nearly all of the critical hepatic functions are carried out by these cells. Indeed, in every parameter of hepatic function examined has shown that the zebrafish liver

performs the same functions ascribed to the mammalian liver, making this an excellent system to study hepatic physiology. There are many other structural and functional similarities between fish and mammals: the biliary network composed of biliary epithelial cells arranged in a ductal system transports bile from hepatocytes to the gall bladder, a unique vasculature with a fenestrated endothelium facilitates transport of molecules from the blood to the hepatocytes, a set of resident immune cells and the hepatic stellate cells, which respond to injury to form a fibrotic scar. All of these cell types, with the exception of Kupffer cells, the resident hepatic macrophage, have been identified in zebrafish. Table 1 outlines the transgenic lines commonly used to mark each of the hepatic cell types.

Work using zebrafish has uncovered many of the genes and pathways that are essential for hepatocyte and biliary development and this has been extensively reviewed (Chu and Sadler, 2009; Goessling and Stainier, 2016). We provide an overview of the fundamental aspects of liver development that have been discovered using zebrafish with a focus on work showing that defects in biliary development can give rise to disease.

2.1 The origin of hepatic cell types

Liver development in all species proceeds through three general phases: progenitor specification, cell type and tissue differentiation, and outgrowth (Figure 1). While this process proceeds much faster in zebrafish, where it is complete in a few days compared to a few weeks in mammals, where it takes a few weeks, the genes and pathways that control each step are largely conserved (Chu and Sadler, 2009; Goessling and Stainier, 2016).

Hepatic specification in zebrafish begins early in development after the formation of the foregut endoderm at 24 hours post fertilisation (hpf). The foregut endoderm is initially located at the embryonic midline and subsequently aggregate into a liver bud left to the midline (Field et al., 2003). During specification, hepatoblasts, the liver progenitor cells, are specified by the expression of two key transcription factors: *hhx* and *prox1* (Ober et al., 2006; Wallace et al., 2001). Hepatoblasts are bi-potential and give rise to hepatocytes and to cholangiocytes, the biliary epithelial cells that form the intrahepatic and extrahepatic bile ducts. Differentiation of hepatoblasts into mature hepatocytes and biliary epithelial cells begins between 50–60 hpf. Genes that participate in key hepatocyte functions, such as *gc* (Noel et al., 2010), *fabp10a* (Venkatachalam et al., 2009) and ceruloplasmin (Korz et al., 2001b) are first detected during hepatocyte differentiation, which begins between 30–48 hpf (Chu and Sadler, 2009; Goessling and Stainier, 2016). Of note, the *fabp10a* promoter (also called *L-fabp* (Her et al., 2003)) is an invaluable resource used by the zebrafish community to drive high expression of transgenes specifically in hepatocytes (see Table 1).

The biliary system in zebrafish is analogous to that of mammals, with a few unique features which are largely based on the difference in hepatic architecture (Yao et al., 2012) and the presence of a preductal epithelial biliary (PDEC) cell in zebrafish. PDECs connect the bile canaliculi of hepatocytes to the smallest branches of the biliary network, analogous to the Canal of Hering in mammals. PDECs then form larger bile ducts to transport the bile produced by the hepatocytes out of the liver (Hardman et al., 2007; Yao et al., 2012). PDECs are too small to see histologically, but ductal structures distinguishable by 70 hpf by staining (Lorent et al., 2004) and through use of transgenics (Table 1). *Tg(keratin18:GFP)* highlights

the forming of intra- and extrahepatic ducts from 3 dpf onwards (Wilkins et al., 2014) and the reporter line that turns on GFP in cells with active Notch signaling (*Tg(Tp1bglob:eGFP)*; (Parsons et al., 2009)) highlights the developing biliary network as early as 45 hpf (Lorent et al., 2010). The preductules formed by PDECs can transport bile efficiently to the gallbladder (Farber et al., 2001) as early as 4 dpf.

Concomitant with the formation of the biliary network, vascular endothelial cells form a complementary network of their own (Figure 1). This essential vascular network for this developing organ also provides instructive architectural cues to the developing hepatocytes and biliary cells. Hepatic endothelial cells originate from the adjacent posterior cardinal vein (Hen et al., 2015) and migrate to first surround by 54 hpf and subsequently invade the liver resulting in the formation of hepatic sinusoids and vessels by 72 hpf. Imaging of endothelial *Tg(kdrl:eGFP)* expression suggests that the endothelial cells migrate along the basal side of hepatocytes to form a branched vascular network by 80 hpf, coinciding with and promoting hepatocyte polarisation (Sakaguchi et al., 2008). This intimate interaction between hepatocytes and endothelial cells allows for maximal exchange area between the blood and hepatocytes, an aspect of hepatic architecture that is important for the hepatocytes serving as the first pass at metabolism of compounds that arrive at the liver immediately after being absorbed in the gut. In mammals, hepatoblasts delaminate from the foregut epithelium and migrate as cords into the mesenchyme that is subsequently invaded with endothelial cells and hematopoietic progenitor cells to form the liver bud (Medlock and Haar, 1983). Analysis of zebrafish mutants with liver specification defects has uncovered a requirement for endothelial precursors during early liver development, which is conserved in mouse (Field et al., 2003; Korzh et al., 2008; Matsumoto et al., 2001; Sakaguchi et al., 2008).

Prospective hepatic stellate cells (HSCs; see Figure 1), expressing *Tg(hand2:eGFP)*, invade the liver from the periphery and move along the developing vascular network starting around 68 hpf (Yin et al., 2012). Although HSCs associate with the vascular network, analysis of *cloche* mutants lacking sinusoidal endothelial cells (Stainier et al., 1995) revealed that they are dispensable for HSC invasion into the liver and differentiation (Yin et al., 2012). Whether HSCs are of neural crest, endothelial, or myofibroblast / lateral plate mesoderm (LPM) origin has been debated for a long time. Recent lineage tracing experiments in mouse revealed that HSCs are generally mesoderm-derived (Asahina et al., 2009). This was confirmed and further refined by work in zebrafish showing that HSCs are derived from the LPM and not endothelial cells, as *Tg(hand2:eGFP)*-expressing HSCs are present in *cloche* mutants (Yin et al., 2012). Thus, the interaction between several cell types is required for proper liver formation

The differentiation of the hepatic architecture is largely completed by 5 days-post-fertilization (dpf, (Lorent et al., 2004; Pack et al., 1996)) while organ growth continues until adulthood. At 5 dpf, the liver takes up metabolic functions (Farber et al., 2001; Howarth et al., 2013) and liver development is essentially complete. A detailed timeline of key cellular processes driving differentiation of the liver architecture is shown in Figure 1.

2.2 Key decisions: Liver or pancreas?

The liver and pancreas share common progenitors in the anterior foregut endoderm. As discussed in Chapter 9 of this volume by Prince *et al.* (*Zebrafish pancreas development and regeneration: Fishing for diabetes therapies*), the pancreas and liver both derive from foregut endoderm and cell fate in the liver is determined by both intrinsic and extrinsic factors that promote hepatic lineage and suppress the pancreatic fate. Single cell lineage-tracing experiments in zebrafish using caged fluorescent dyes in six- to eight- somite stage embryos showed that the most lateral foregut cells give rise to liver and ventral/exocrine pancreas, while more medial cells will form the intestine and dorsal pancreas (Chung et al., 2008). Likewise, bi-potential hepatopancreatic progenitors were identified in the mouse by embryonic tissue explant experiments and single cell lineage tracing (Deutsch et al., 2001; Miki et al., 2012; Rossi et al., 2001; Tremblay and Zaret, 2005).

Vertebrate liver specification represents a two-step process, in which anteroposterior patterning events in the early embryo establish in a broad endodermal region the competence to adopt a hepatic fate and the subsequent release of inductive signals by a tightly controlled signalling cascade. Hepatic competence has primarily been studied in mice, where pioneer transcription factors that bind chromatin and make it accessible for lineage-specific transcription factors have been extensively studied (Zaret and Mango, 2016). Two such pioneering transcription factors that have been studied in detail in zebrafish are Gata4 and Gata6. Depletion of either these factor results in significantly smaller liver buds, while the double knockdown of both factors leads to the complete absence of hepatic *transferrin* expression, a marker of differentiated hepatocytes (Holtzinger and Evans, 2005). Bmp signalling promotes hepatic competence in vertebrates, in part by regulating Gata expression (Shin et al., 2007). In zebrafish, *bmp2b* is expressed in the LPM adjacent to the foregut endoderm preceding hepatic specification, and hepatoblast *hhex* and *prox1* expression is reduced when Bmp signalling is blocked during this period (Chung et al., 2008; Shin et al., 2007). Intriguingly, overexpression of *bmp2b* increases hepatoblasts at the expense of Pdx1-expressing ventral pancreas and intestinal progenitors (Chung et al., 2008), providing strong support for the fate switching functions of Bmp signalling. Subsequent transcriptome analysis uncovered the transcription factor *four and a half LIM domains 1b (fh11b)* as a target of Bmp2b signalling and a critical mediator of its role in promoting hepatic fate versus the pancreas fate choice (Xu et al., 2016). The description of how *pdx1* promotes pancreatic fate is described in Chapter 9 of this volume.

2.3 Delivering a fully formed organ

Both intrinsic and extrinsic signals are critical for formation of all organs, as discussed in nearly all chapters in this volume. In the liver, a breakthrough discovery identified liver inductive signals following the establishment of hepatic competence in the early embryo through a forward genetic screen where the *prometheus/wnt2bb* mutant was discovered to have severely impaired hepatoblast formation (Ober et al., 2006). Other work showed that enhanced Wnt signalling in *apc* mutants or via Wnt2bb/Wnt8 overexpression induces ectopic liver progenitors at the expense of ventral pancreas and intestinal progenitors, placing Wnt signalling at the center of the hepatic induction phase of liver development (Goessling et al., 2008; Poulain and Ober, 2011; Shin et al., 2011). Wnt signalling, which is

regulated by Hnf1b and EpCam activity during hepatoblasts specification (Lancman et al., 2013; Lu et al., 2013), is suppressed prior to hepatic induction (Goessling et al., 2008). It is then activated through Wnt2bb/Wnt8 during hepatic induction, and further activated through additional ligands to promote proliferation of hepatoblasts following hepatic specification (Poulain and Ober, 2011). In support of this paradigm, *wnt2bb* is expressed in the LPM after *bmp2b* (Chung et al., 2008; Ober et al., 2006), supporting the model where Bmp signalling has a permissive function and Wnt signalling has an inductive function during hepatic specification (Goessling and Stainier, 2016).

Cytokine prostaglandin E₂ (PGE₂) signalling likely also has switch-like activity upstream of Bmp2b (Nissim et al., 2014). PGE₂ receptors *ep2a* and *ep4a* are expressed bilaterally in the foregut domain during early somitogenesis stages and *bmp2b* expression is reduced when these receptors are depleted. Intriguingly, treatment of mouse embryonic stem cells with high or low amounts of PGE₂ leads to fate switching between liver and pancreas differentiation (Nissim et al., 2014). This hepatogenic potential of PGE₂ signalling similarly promotes liver regeneration following partial hepatectomy in zebrafish and mouse (Goessling et al., 2009). Additional work from the Goessling group demonstrated that nuclear receptor subfamily 5, group A, member 2 gene (*nr5a2*) is also important for determining liver-pancreas fate. By using a combination of morpholinos, mutants and drugs to target Nr5a2 in developing zebrafish, they identified a requirement during exocrine, but not endocrine, pancreas development, and for liver growth (Nissim et al., 2016). This suggests that Nr5a2 has different roles at different states of development, first, in priming the hepatopancreatic progenitors, and then in their outgrowth.

These findings are not only important for providing a full understanding of liver development, but are also of practical utility, as they can be utilized to design effective protocols to promote hepatic fate. Similar to work described in Chapter 9 of this volume where factors that drive pancreas development have been used to generate pancreatic beta-cells *in vitro*, the progress in understanding the molecular switches regulating this early fate decision has influenced protocols to differentiate mammalian embryonic or induced pluripotent stem cells into hepatocytes. In these protocols, many of the above described key signalling events occurring in embryonic liver development have been recapitulated (Gouon-Evans et al., 2006; Nissim et al., 2014; Si-Tayeb et al., 2010). Thus, work on the development of multiple organs can be combined and translated to clinical utility.

2.4 Organ asymmetry: positioning the liver in the body

A hallmark of the liver and other internal organs is their asymmetric arrangement with respect to the anteroposterior body axis allowing their efficient packing within the body cavity. Hepatoblasts are specified at the midline of the embryo and are placed on the left side in zebrafish and on the right side in mammals. We will focus on work in zebrafish that has established a detailed model of vertebrate liver positioning.

Seminal experiments in zebrafish embryos first identified the asymmetric migration of the bilateral neighboring LPM as a critical step for the leftward positioning of hepatoblasts and gut looping (Horne-Badovinac et al., 2003). During gut looping, left LPM moves dorsal of the endoderm and the right LPM moves ventrolateral. In *heart and soul* mutants (Stainier et

al., 1996), where protein kinase C, *iota* (*prkcι*) is inactivated, the epithelial structure of the LPM and its asymmetric movement are disrupted, resulting in a midline liver and gut (Sakaguchi et al., 2008). Likewise, mutants with impaired extracellular matrix (ECM) remodeling, specifically, laminin degradation, display defective LPM migration and midlineivers (Hochgreb-Hägele et al., 2013; Yin et al., 2010). Together, these findings suggest that as the LPM migrates right, it displaces the midline endoderm to the left by a motive force.

We found that live imaging of sparsely labeled hepatoblasts revealed that they migrate actively to form the liver bud on the left and that their directionality is provided by the repulsive activity in the right LPM (Cayuso et al., 2016). These cell-cell interactions involve long cell protrusions and are coordinated by EphrinB1 and its receptor EphB3b, located in hepatoblasts and the LPM respectively. The precise contributions of active hepatoblast migration and the LPM's pushing force to liver positioning remain to be determined. Another study used chemical screening identified retinoic acid and specifically its receptor Rargb in control of organ laterality as early as specification stages (Garnaas et al., 2012), which has also been shown to play a role in laterality in mammals (Yasui et al., 1998)

These phenotypes are highly reminiscent of human disorders such as heterotaxy (OMIM: 601086), in which the majority of internal organs are misaligned. The consequences can be disastrous: patients have cardiac defects (as discussed in Chapter 1), cystic kidney (discussed in Chapter 2) and some have biliary defects. Work using zebrafish has provided insight into the basic developmental biology of the liver and other endodermal organs has influenced stem cell protocols and has laid the foundation for identifying molecular mechanisms underlying left-right organ morphogenesis and associated disorders.

2.5 Liver outgrowth

A number of aspects contribute to liver growth and size such as signaling pathways promoting proliferation, DNA replication and the regulation of cell survival at the level of progenitors as well as differentiated cell types. A working hypothesis in the field is that there are some shared factors that control hepatic outgrowth in embryos and liver regeneration in adults and that some of these factors may be co-opted by hepatocytes as they are transformed to malignant cells during hepatocarcinogenesis.

Following specification of the progenitor cell pool, the appropriate number of cells to form the full organ is dictated by the concerted action of Wnt, prostaglandin PGE₂, and nitric oxide signaling promoting proliferation (Cox et al., 2014; Goessling et al., 2008; Nissim et al., 2014; Poulain and Ober, 2011). In parallel, Id2a maintains hepatoblast number (Khaliq et al., 2015). Id2 controls hepatoblast proliferation and also promotes cell survival independent of proliferation through two downstream targets: Anxa4 and Nexin7 (Zhang et al., 2014). Loss of function mutants revealed that biliary cell survival requires Snapc4 (Schaub et al., 2012a), while a set of genes required for mitochondrial processes and homeostasis, including *tomm22*, *subv3L1*, *trx2* and *mtch2*, are vital for hepatocyte survival (Curado et al., 2010; Landgraf et al., 2016; Schaub et al., 2012a; Zhang et al., 2014). Whether these same genes are important in maintaining cell survival in adults is not yet known.

Understanding the mechanisms by which organ-specific cell regulates proliferation remains a central unanswered question in developmental biology. Given the complexity of cell proliferation, a significant number of factors have been identified mediating this cellular process at different levels. The transcription factors Cebpa and Klf6/Copeb regulate cell proliferation, with or without cell death (Yuan et al., 2015; Zhao et al., 2010). The Def gene controls G1 to S-phase transition and small livers observed in mutants are due to cell cycle arrest (Chen et al., 2005). The loss or impaired function of several chromatin remodeling factors including Hdac1, Hdac3 and Ssrp1a results in small livers by 5 dpf generally due to reduced cell proliferation and DNA replication defects (Anderson et al., 2009; Farooq et al., 2008; Koltowska et al., 2013; Noel et al., 2008; Sadler et al., 2007). Our work has also found that cell cycle arrest due to widespread epigenetic changes is important for hepatic outgrowth. Mutation of *the ubiquitin like with phd and ring finger domains 1* gene (*uhrf1*) does not affect liver specification (Sadler et al., 2007), but during outgrowth, arrests hepatocytes in S-phase of the cell cycle (Jacob et al., 2015). Uhrf1 functions as a co-factor for DNA methyltransferase 1 (*dnmt1*) to copy the pattern of DNA methylation during cell division (Bostick et al., 2007; Sharif et al., 2007). We showed that *uhrf1* mutant hepatocytes continually replicate their DNA, yet fail to divide and ultimately undergo apoptosis. Mutation in *dnmt1* shows the same phenotype (Jacob et al., 2015), indicating that reprogramming of the DNA methylome is the likely underlying mechanism of this unusual cell cycle phenotype.

Compelling evidence from zebrafish indicates that key genes involved in development also cause liver cancer. The involvement of the Hippo pathway in regulation of hepatic size and in liver cancer has been intensely studied in mice (Yimlamai et al., 2015) and a recent study in zebrafish provides compelling evidence that Yap signaling promotes liver growth by supplying the cellular building blocks enabling cell proliferation, leading to both hepatomegaly and liver tumors in adults (Cox et al., 2016). Thus, in both the developing liver and liver cancer, the hippo pathway is essential. Similarly, our work has shown that while loss of *uhrf1* blocks liver development (Sadler et al., 2007), overexpression of human UHRF1 in zebrafish hepatocytes causes liver cancer (Mudbhary et al., 2014). It will be interesting to determine if these pro-growth and epigenetic pathways cooperate to promote liver cancer.

We speculate that many of the factors described here are also important for the continuous growth of the liver during larval maturation and adulthood. Several of these factors have been shown to also control liver regeneration in adult zebrafish and mammals, highlighting the parallelism between hepatic outgrowth in embryos and regeneration in adults.

2.6 Biliary development

When the liver anlage is established, bi-potential hepatoblasts differentiate into hepatocytes and biliary epithelial cells (BECs). Notch signaling plays an important and recurrent role in the formation of the tubular biliary network (Lorent et al., 2010; Lorent et al., 2004). Knockdown of Notch receptors or Jagged ligands at early stages of biliary cell development inhibits the formation of BECs leading to bile duct paucity (Lorent et al., 2004), similar to the dominant Jagged1 phenotypes in human Alagille Syndrome (OMIM: 118450), discussed

below. Experiments to inhibit Notch during discrete developmental times showed that Notch regulates the remodeling of BECs into a functional network, including the formation of hepatocyte bile canaliculi (Lorent et al., 2010). Specifically, the assembly of specified BECs into a functional network requires Sox9b, which acts both as a target and mediator of Notch signaling (Delous et al., 2012; Manfroid et al., 2012; Xu et al., 2016). Biliary network malformations observed in *sox9b* mutant larvae do not recover and adult mutants exhibit signs of cholestasis with bile accumulated in liver and pancreas (Deutsch et al., 2001).

In addition the transcription factors Onecut1/Hnf6, Onecut3, and Vhnf1 (Matthews et al., 2008; Matthews et al., 2004) are all integral to development of the zebrafish hepatobiliary network, as in mammals (Lemaigre, 2009). The establishment of cell polarity and junctions as critical for hepatobiliary development in mammals (see review by (Lemaigre, 2009) and work with zebrafish mutants confirm this conserved requirement for tight junctions in biliary development. Mutation of the tight junction protein *claudin15-like b* in zebrafish causes abnormal biliary ducts, canalicular malformations, and impaired hepatocyte polarity (Cheung et al., 2012).

Subsequent maintenance of the biliary network is regulated by Snapc4, a subunit of a small nuclear RNA activating complex (Schaub et al., 2012b). As hepatocytes seem unaffected in *snapc4* mutants, indicating that the function of Snapc4 is specific to biliary cells. In many of these studies, live imaging of GFP-expressing biliary cells in cultured livers or in embryos provided invaluable insights into cell behaviors driving this dynamic morphogenetic process (Lorent et al., 2004; Ningappa et al., 2015).

In summary, despite the relative simplicity of the liver, the coordinated development of the different hepatic cell types is complex. By integrating studies in zebrafish with other models, including rodents and reptiles, we are achieving a deeper understanding of how the liver is patterned, how fate choices are made, and how liver growth is controlled. In the next section, we discuss how similar paradigms are being studied in the context of liver regeneration.

2.7 Biliary disease: Alagille syndrome and beyond

Biliary atresia (BA) is a rare pediatric disease typically diagnosed in infancy that is caused by progressive obliteration of the bile ducts. This is the best-characterized relationship between a developmental defect and hepatobiliary disease. Loss of bile ducts results in accumulation of bile in the liver, which is highly toxic to hepatocytes, and BA is fatal without transplant. The prevailing theory in the field is that a failure in bile duct development is the fundamental cause of BA (Asai et al., 2015). Alagille syndrome is an inherited cause of BA that is also associated with cardiac and skeletal malformations caused by mutation in Jagged-1. Extensive studies in humans, mice and zebrafish have demonstrated how Jagged/Notch signaling pathway functions in biliary specification and differentiation and a subset of BA patients have mutations in one of the genes in this pathway (Flynn et al., 2004; Kodama et al., 2004). There are several tools to study the zebrafish biliary system (Table 1); most of them utilize confocal imaging of the liver followed by 3-D rendering to obtain a comprehensive view of the biliary network.

Other human genes that are important for biliary disease have also been identified through work in zebrafish. Sequencing of the zebrafish genome revealed that about 70% of human genes have at least one obvious zebrafish counterpart, and this number increases to 82% when focusing on human disease-related genes (Howe et al., 2013). Together with the ease for studying gene function and the high morphological and molecular similarity of tissue and organ development in zebrafish to that in humans makes zebrafish an ideal system for testing candidate disease factors identified by Gene Association Studies (GWAS). This has been particularly successful when studying genes regulating biliary development.

GWAS studies have identified several candidate genes for BA, including Adducin 3 (*ADD3*) and X-prolyl aminopeptidase 1 (XPNPEP1). A recent study in zebrafish found that only depletion of *add3* caused biliary defects similar to BA, prioritizing *ADD3* as a BA susceptibility gene over XPNPEP1 (Tang et al., 2016). Another study of Caucasian BA cases identified a susceptibility locus on human chromosome 14q21.3, including the *ARF6* gene (Ningappa et al., 2015). The two zebrafish homologs, *arf6a* and *arf6b* are expressed in the liver and knock down experiments showed they are required for early biliary development. In another study, knockdown of *vsp33b* and *vipar*, genes commonly mutated in arthrogryposis, renal dysfunction and cholestasis syndrome (ARC) patients in zebrafish reproduced the biliary defects observed in ARC patients and established *vsp33b* as a driver of this disease (Cullinane et al., 2010; Matthews et al., 2005). Finally, analysis of over 60 BA patients identified glypican 1 (GPC1) as a BA susceptibility gene, and BA patients have reduced GPC1 expression. The finding that bile duct development was defective in zebrafish *gpc1* morphants (Cui et al., 2013b) provides support to this as a candidate gene for BA. However, many BA patients have no detectable mutations in any of the genes described above, and a second theory is that a viral infection prevents biliary development.

Support for the viral basis of BA came from studies in zebrafish which found that activation of the interferon response in the liver was sufficient to cause both a reduction of number and shortening of biliary branches (Cui et al., 2013a; Matthews et al., 2011). The interferon response was induced by injecting zebrafish larvae with a drug (5-azacytidine) that both blocks DNA methylation and causes DNA damage, and examination of samples from BA patients suggests that some have reduced DNA methylation in the biliary cells that remain in these diseased livers (Cui et al., 2013a; Matthews et al., 2011). Together, these data support a compelling hypothesis that either an intrinsic defect in the ability of biliary cells to faithfully copy their DNA methylome during cell division or that an external agent, such as an environmental toxin (see chapter 9 of this volume by Bambino and Chu) can mimic a viral infection and potentially promote immune mediated destruction of biliary cells.

One of the most exciting studies in recent years in the field of biliary disease was carried out using zebrafish to identify a naturally occurring plant compound that can destroy the extrahepatic biliary duct (Lorent et al., 2015). Reports in the livestock literature indicated that in regions of Australia, lambs born to mothers who ate a specific plant would develop fatal BA. A team of basic and translational investigators set out to identify the toxic compound produced by this plant. Using zebrafish to screen extract from these plants, the damaging compound was identified as 4-methoxy-*seco*-betavulgarin, which they termed Bilatresone (Lorent et al., 2015). Transcriptional profiling of livers with Bilatresone-

induced liver injury revealed an increased expression of genes associated with redox stress (Zhao et al., 2016). A fluorescent sensor showed that extrahepatic biliary duct cells are particularly sensitive to redox stress in zebrafish, supported by a parallel study in mouse providing synergistic results (Waisbourd-Zinman et al., 2016; Zhao et al., 2016). Together, both studies outline a possible mechanism for human pathologies and highlight the power of cross-species investigations. Intriguingly, the mechanism of Bilatresone action may hold the key to identifying how other toxins or infections, which are more common in humans, can cause biliary disease. In summary, BA represents the most common liver disease caused by developmental defects in the liver. Studies in zebrafish have provided not only an optimal system to test the functional relevance of candidate BA genes, but also as a system to discover new pathways that are important for biliary development and for BA.

3. Liver regeneration

The liver is the body's primary defense against endogenous and environmental stressors, and as such, it harbors an amazing intrinsic ability to regenerate upon injury. Mammals are fantastic failures when it comes to regeneration, with the liver among the few exceptions. In contrast, zebrafish easily regrow damaged or resected organs including the heart, pancreas, spinal cord and fins (Gemberling et al., 2013). The features distinguishing organisms with robust regenerative capacity from mammals, who are utter failures at organ regeneration, is intensely investigated (Stoick-Cooper et al., 2007). A major goal regenerative medicine is to manipulate mammalian cells so that they can be more like fish and repair or replace damaged organs.

3.1 Mechanisms of liver regeneration

The almost unlimited proliferative capacity of hepatocytes is central to liver regenerative potential. Hepatocytes are quiescent under normal physiological conditions but can be stimulated to grow and regain liver function when the liver is damaged and new liver mass is needed to maintain homeostasis. This property is demonstrated through serial hepatocyte transplantation experiments using rodent models of liver injury (Overturf et al., 1997; Rhim et al., 1994) whereby mature hepatocytes repopulate the liver. This stands in stark contrast to tissues like skin or gut, where lineage-specific stem cells proliferate and differentiate to replace the loss of mature cells. However, in response to severe liver injury that renders hepatocytes incapable of efficient division, it is thought that bi-potential hepatobiliary progenitor cells are activated to replace the damaged liver. This response has recently been elegantly investigated in zebrafish (Choi et al., 2014; He et al., 2014). Some evidence shows that parenchymal cells can be induced to transdifferentiate to regenerate functional hepatocytes (Michalopoulos and Khan, 2015), yet it is unclear whether this mechanism of regeneration is conserved across species.

Decades of studies on liver regeneration in mammals have revealed a complex network of mitogens and downstream signaling pathways as essential for sustaining liver function and stimulating proliferation when hepatocytes are compromised or their mass is reduced. This has been extensively reviewed (Michalopoulos, 2014; Taub, 2004) and Figure 2 summarizes the basic mechanisms of liver regeneration in mice and zebrafish. We discuss the common

mechanisms that guide liver regeneration in vertebrates where zebrafish research has provided unique insights.

Compared to rodent models, zebrafish embryos and larvae provide several advantages such as ease of manipulation, state-of-the-art imaging, the ability to carry out chemical screens and generate mutants and transgenics (Table 1). These characteristics of zebrafish have been applied to the study of liver regeneration, and provided a unique lens with which to view this well studied and complex process. Enhancing the ability of the liver to regenerate, in turn, could improve outcomes for patients awaiting transplant, for those receiving a small-for-size transplant, and could enhance the regrowth of the remaining liver in living donors.

3.2 Stimulating regeneration: Cut or kill

Since the hepatocyte proliferative capacity can be exhausted in end-stage liver failure, an alternative to patient derived hepatocytes is sought. Two potential different mechanisms utilized by the liver to regenerate lost mass have been identified through work in zebrafish and mammals, outlined in Figure 2. First, hepatocytes serve as the source of more hepatocytes, and can be stimulated to proliferate. The fact that fully differentiated hepatocytes can in effect serve as their own stem cells under most physiological conditions suggests that replacing failed livers with mature hepatocytes could augment liver function.

A number of experimental approaches have been developed in mammals and zebrafish to study liver regeneration. These can be classified into two categories: 1) surgical or 2) injury-induced, and a few studies combining both methods. In surgical models, parts of the liver are surgically removed to trigger hypertrophy and proliferation of hepatocytes in the remnant lobes (Higgins and Anderson, 1931). In injury-induced models of liver regeneration, liver injury is introduced chemically, genetically, or by a combination of both. In chemically-induced injury models, animals are exposed to hepatotoxins that trigger cell death in the liver followed by proliferation of remaining hepatocytes to replace dead cells. Similar principles are adopted in genetically-induced liver injury models; where instead of chemicals, engineered genetic defects in hepatocytes produce continuous or trigger-induced cell death that lead to heightened hepatocyte proliferation. While injury-induced models more closely resemble the etiology of liver disease patients, surgical models provide a simplified model of regeneration that arguably led to the most mechanistic insights into a highly complex biological process.

3.2.1 Liver resection in Mammals—One major advantage of the surgical model is the nearly century old observation that following partial hepatectomy, the remaining hepatocytes synchronously emerge from a state of quiescence and re-enter the cell cycle (Higgins and Anderson, 1931). Within minutes of removing 70% of the liver mass is removed in mammals, the enormous change in blood pressure due to loss of tissue mass initiates a series of signaling pathways, including induction in urokinase activity, Wnt/ β -catenin and notch signaling (Michalopoulos, 2010). Within hours, cytokine and growth factors are activated to stimulate hepatocyte exit from quiescence (G_0) to enter the cell cycle.

Of all the cytokines that have been investigated, interleukin-6 signaling alone controls the expression of ~36% of over 100 “immediate-early genes”, a unique gene signature that

characterize this early regenerative phase (Li et al., 2001). In a simplified system of cultured primary hepatocytes, a number of growth factors can promote hepatocyte proliferation including hepatocyte growth factor, ligands of epithelial growth factor receptors such as epithelial growth factor, transforming growth factor alpha, amphiregulin, hepatocyte growth factor and heparin-binding EGF-like growth factor and fibroblast growth factors. These have been extensively studied. At around 24–30 hours post-hepatectomy, hepatocytes begin to prepare for S phase by expressing DNA replication-related genes under the control of the retinoblastoma protein and various cyclins, which is followed by maximum DNA synthesis at around 48 hours post-hepatectomy. In this model, each hepatocyte proliferates roughly 1.6 times on average to make up for lost liver mass. A recent study of the response to removing different amounts of mouse liver discovered two distinct regenerative strategies adopted by the liver: following 30% hepatectomy, hepatocytes mainly expand in size to make up for the lost liver mass, whereas following 70% hepatectomy, hepatocytes both hypertroph and proliferate to make up for the lost mass (Miyaoaka et al., 2012).

The physiological, metabolic and hemodynamic changes that occur following partial hepatectomy are rapid and dynamic. This is a complex process that despite intense investigation by teams of outstanding scientists over several decades, a master regulator of regeneration has yet to be identified. This suggests that there is no “magic bullet” for promoting regeneration. Instead, attention is now focused on defining a strategy to improve regeneration that relies on coordinated manipulation of multiple pathways.

3.2.2. Liver resection in Zebrafish—The impressive capacity to undergo epimorphic regeneration in several tissues such as fin, heart and liver in zebrafish stands in stark contrast to mammals, where few organs can regenerate. In contrast, mammals regenerate the liver by compensatory regeneration, whereby hepatocytes proliferate in the remaining lobes of the liver. Compensation by moderate enlargement of the remaining lobes has been also documented in zebrafish, so that in this system, regeneration occurs by epimorphic regrowth of the resected lobe and compensatory enlargement of the remaining lobe to restore liver mass by 7 days following surgery (Goessling et al., 2008; Kan et al., 2009; Sadler et al., 2007).

Consistent with the critical role of WNT signaling in both development and cancer, this pathway is essential for hepatocyte proliferation during liver regeneration in both mammals and zebrafish. Mice deficient in β -catenin have decreased markers of cell proliferation after partial hepatectomy (Tan et al., 2006). In line with these findings, zebrafish mutants deficient in Wnt signaling have reduced liver progenitor populations, whereas increased Wnt signaling as a result of *apc* mutations in zebrafish promoted both hepatic specification and liver regeneration in adult zebrafish following partial hepatectomy (Goessling et al., 2008). A chemical screen using zebrafish embryos identified a non-selective Cox inhibitor, indomethacin, which inhibited Wnt signaling and blocked liver specification in embryos and regeneration in adults through the suppression of PGE₂ production (Goessling et al., 2009; North et al., 2007). This suggests a novel link between inflammation, PGE₂ accumulation, and cellular proliferation. This work also highlights the reiterative use of pathways in liver development and regeneration.

The high level of conservation of the regenerative process exhibited by mouse and zebrafish livers following partial hepatectomy allowed investigators to apply zebrafish genetics to test the *in vivo* contribution of several pathways that have thus far only been shown to regulate hepatocyte proliferation in cell culture systems. Striking examples are transgenic zebrafish where transient and inducible overexpression of dominant negative variants of fibroblast growth factor receptor (Fgfr) or bone morphogenetic protein receptor (Bmpr) that can almost completely block signaling through these receptors. With this strategy, Kan et al were able to overcome both the challenges of ligand/receptor multiplicity and establish the importance of Fgfr and Bmpr signaling in liver regeneration. Blocking either Fgfr or Bmpr signaling dramatically impaired liver regeneration in zebrafish following partial hepatectomy (Curado and Stainier, 2010; Kan et al., 2009). These pathways are also essential for liver specification during development (Zaret and Mango, 2016) and promote proliferation in cultured hepatocytes (Taub, 2004) highlighting these as common mechanisms that regulate liver growth under physiological conditions.

3.2.3. Killing hepatocytes in mammals using toxins and genetics—Hepatic failure occurs when the replicative capacity of hepatocytes is insufficient to replace massive damage. If the liver suffers widespread damage and hepatocyte proliferation is limited or if the hepatocytes loss is so massive that the remaining cells cannot adequately and quickly repopulated the liver, an alternative mechanism to regenerate lost mass is activated: bi-potential liver progenitor cells that can differentiate into both cells of the biliary and the hepatic lineage (i.e. oval cells in mammals) are activated to repopulate the liver (Michalopoulos and Khan, 2015). In this scenario, liver regeneration is dictated not only on hepatocyte proliferation but also on the degree of continuous cell death, on the extent of fibrosis, which may physically restrain liver growth, and on the ability to mobilize progenitor cells. We review the extensive use of mouse models of fulminate hepatic failure here and then discuss how work in zebrafish has extended the understanding of progenitors and regeneration (see Figure 2).

Carbon tetrachloride is the most frequently used chemical to induce liver injury in mammals, as a single dose induces massive hepatocyte necrosis within 24 hours (Wong et al., 1998), which is efficiently repaired by replacement of the damaged hepatocytes. Repeated doses cause chronic hepatic injury liver with the associated inflammation, fibrosis, and activation of the immune response. Thus, chemically-induced liver injury creates an environment that more closely resemble patients with chronic liver diseases, but use of this approach is complicated by the myriad of factors inherent in the response. In addition, systemically applied chemicals such as carbon tetrachloride may have off-target effects on non-liver tissues that indirectly influence the regenerative response in the liver.

Genetically-induced liver injury models ameliorate both off-target effects and the non-uniform distribution associated with chemically-induced liver injury. In both mice and humans with fumarylacetoacetate hydrolase (FAH) deficiency (OMIM: 613871), the lack of this critical enzyme for tyrosine metabolism in hepatocytes leads to accumulation of toxic metabolic intermediates and massive liver injury. If the hepatocytes are not replaced, mice die within 12 hours after birth (Grompe et al., 1993) unless the production of the toxic metabolite is blocked with a drug. However, when the drug is removed, all hepatocytes die

and the mice succumb to liver failure (Grompe et al., 1995) unless *Fah* expressing hepatocytes competent to engraft and proliferate are reintroduced to the liver. This model serves as a template to introduce alternative sources of hepatocytes to evaluate the ability of grafting and repopulating the liver and rescuing the mouse. This system allows direct comparison of the *in vivo* regenerative potential of the different cell types in the liver and establishes the available sources of new hepatocytes to regenerate the liver under different stressors.

In the *Fah* deficient mouse model, lineage tracing of donor cells revealed that almost all the hepatocytes that repopulated the *Fah*^{-/-} mouse liver originated from hepatocytes whereas only <1% originated from Sox9+ liver stem cells (Tarlow et al., 2014). This is supported by lineage tracing experiments in a range of chemically induced liver damage (Schaub et al., 2014; Yanger et al., 2014). *In vivo* lineage tracing experiments in another case of severe liver damage in *Mdm2* deficient mice, showed that liver was regenerated by expansion of liver progenitor cells (Lu et al., 2015). Thus, progenitors are a likely source of new hepatocytes in cases where regeneration cannot be accomplished by hepatocytes alone.

A unique attribute of hepatocytes suggested to promote their proliferative capacity is their ploidy. An elegant study using *Fah* deficient mice demonstrated that under stressed conditions the different ploidy states of hepatocytes, dubbed the “ploidy conveyor”, provide an additional source of genetic diversity that can be selected for to promote organism survival (Duncan et al., 2012; Duncan et al., 2010). It is not clear whether zebrafish utilized this same mechanism, as hepatocyte ploidy has not been thoroughly investigated. It will be interesting to see whether similar mechanisms apply to the handful of other cell types in mammals that demonstrate polyploidy (Schoenfelder and Fox, 2015).

3.2.4. Progenitor mediated regeneration in zebrafish: Seeing is believing—

Studies in zebrafish and mice demonstrated that biliary cells themselves may transdifferentiate into hepatocytes, which may occur through a dedifferentiated stem cell like state. Two of these studies combined the beauty of zebrafish genetics with state-of-the-art imaging techniques to perform *in vivo* lineage tracing experiments that unequivocally established biliary cells as the source of hepatocytes at least in one model of extensive hepatocyte loss.

Figure 3 shows a zebrafish model analogous to the *Fah*^{-/-} mouse, whereby massive hepatocyte death was induced in transgenic zebrafish whereby adding the nontoxic pro-drug metronidazole to transgenic zebrafish that selectively express nitroreductase in hepatocytes (*Tg(fabp10:mChNTR)*) and converts metronidazole into a cytotoxic metabolite, followed by liver regeneration within days (Choi et al., 2014; Curado et al., 2007; He et al., 2014). By crossing *Tg(fabp10:mChNTR)* and other similar lines with transgenic zebrafish that express lineage specific fluorescent markers (Table 1), elegant imaging studies during the entire time period of regeneration performed by two groups showed that transdifferentiation of biliary cells into hepatocytes occurred (Choi et al., 2014; He et al., 2014). Another study showed that this transdifferentiation occurred through transient Wnt and Notch signaling in cells which appeared to be an intermediate hepatobiliary cell (Huang et al., 2014).

Thus, it appears that under normal conditions or mild stress, hepatocytes serve as their own progenitors, but under extreme circumstances when the proliferation of hepatocytes is impaired or insufficient, a progenitor cell population that may be of the biliary lineage can expand to fill that void (Figure 2). By taking advantage of the unique attributes of zebrafish that allow imaging in real time during regeneration, zebrafish has been used to its best advantage to promote discoveries in liver regeneration.

3.3. Epigenetics mechanisms regulate hepatocyte proliferation

Most of the work to identify key genes involved in liver regeneration in mice and zebrafish has relied on manipulation of single genes. However, genome-wide gene expression studies established that the coordinated expression of thousands of genes is required for the liver to regenerate even in the simple partial hepatectomy model (Li et al., 2001; Su et al., 2002). Current data suggest that no single signaling molecule or pathway alone is sufficient to drive this massive change in the gene expression landscape, suggesting that either complex cross-talk between different signaling pathways or yet uncharacterized higher order processes that can influence multiple pathways simultaneously. Epigenetics represent such higher-order processes that can alter the expression of many genes simultaneously, as is found during liver regeneration, yet the role of epigenetics in liver regeneration has only begun to be investigated.

The involvement of epigenetic mechanisms regeneration of the zebrafish fin, arguably the most widely studied model for tissue regeneration in zebrafish have uncovered a role for DNA methylation in this process. For example, *de novo* DNA methyltransferases are induced during zebrafish fin regeneration (Takayama et al., 2014) and DNA hypomethylation occurs early during fin regeneration, which was attributed to active DNA demethylation (Hirose et al., 2013). Whether re-patterning of the DNA methylome is conserved in other regenerating tissues is not known, however a recent exciting study demonstrated that epigenetic regulation of *leptin b* is important for the function of this gene in heart and fin regeneration (Kang et al., 2016). The “tissue regeneration enhancer element (TREE)” that was identified in this study was found to be upstream of other genes with known roles in zebrafish fin (*fgf* effectors) and heart (*nrg1*) regeneration. The fact that TREEs are activated during the regeneration of different tissues and are conserved between zebrafish and mouse suggests they may also be involved in liver regeneration, which represents an exciting future avenue of investigations.

Despite the ability to facilitate massive changes in gene expression profiles such as those found during liver regeneration, very few studies have investigated the involvement of DNA and histone modifications in this process. In fact, the most comprehensive studies that investigated the involvement of epigenetic processes on liver regeneration came from studies using zebrafish. Through a forward genetic screen in zebrafish embryos, we found that *uhrf1* mutants fail in hepatic outgrowth during development and heterozygote mutation for *uhrf1* represses liver regeneration following partial hepatectomy in adults. Transcriptome analysis of *uhrf1* mutants revealed a dramatic across-the-board induction of cell cycle genes that corresponded with cell cycle defects such as aberrant DNA replication, cell cycle arrest in S phase, and cell death, leading to the observed liver growth defect (Jacob et al., 2015).

Moreover, over-expression of Uhrf1 in zebrafish hepatocytes led to cellular senescence followed by uncontrolled cellular proliferation and rapid onset of liver cancer (Mudbhary et al., 2014), placing the epigenetic regulator Uhrf1 and DNA methylation at the center of the regulatory network in control of hepatocyte proliferation. How global changes in DNA methylation preferentially affect the expression of genes controlling cell proliferation during hepatic outgrowth in the developing zebrafish embryo, and whether the same epigenetic mechanisms apply to liver regeneration in adult mammals has yet to be determined.

Further evidence in support of epigenetic factors driving liver regeneration has emerged from work with zebrafish with metronidazole-induced liver injury in *Tg(fabp10a:mChNTR)* zebrafish (Ko et al., 2016). In this study, a screen of 44 compounds with known targets identified two distinct bromodomain and extra-terminal domain (BET) inhibitors that almost completely blocked liver regeneration by preventing conversion of biliary cells to hepatoblasts and to proliferation of hepatoblasts. BET inhibitors which prevent the binding of transcription factors and chromatin modifiers to acetylated histone tails (Shi and Vakoc, 2014). These results suggest that transcription factor binding to acetylated histone tails play important roles in liver regeneration and are reminiscent of the requirement of TREEs during regeneration of the zebrafish fin. The finding that BET inhibitors similarly inhibited oval cell proliferation in mice suggests a conserved role for BET proteins and histone acetylation in the control of liver progenitor cell expansion. These studies highlight how the cutting edge in liver regeneration is accelerated through work in zebrafish and other models.

4. Strengths and challenges of using zebrafish to study liver regeneration

The zebrafish represents one of the simplest vertebrate organisms that can be used to model the human liver. In zebrafish embryos, which conveniently develop *ex vivo*, mature hepatocytes can be observed as early as 72 hpf and the liver is fully functional by 5 dpf (Goessling and Sadler, 2015). This short timeframe for liver development makes zebrafish embryos a very cost-effective model to study liver processes such as liver regeneration. Additionally, fast development combined with the fact that each successful mating produces hundreds of embryos together make the zebrafish model highly adaptable to large-scale unbiased chemical/genetic screen that often yield novel and unexpected insights into the underlying biology, as demonstrated by the surprising discovery of epigenetic regulators in liver regeneration (Ko et al., 2016).

As the liver represents our body's first line of defense against environmental stressors, the scalability of the zebrafish system also provides a unique opportunity to screen single and/or complex mixtures of environmental toxicants for their impact on liver regeneration. Another major advantage of using zebrafish embryos to study liver regeneration lies within the imaging prowess of this model. In addition to wild-type zebrafish having optically transparent embryos, transgenic zebrafish lines have been established that fluorescently label every major cell population in the liver (Table 1), providing a comprehensive toolkit to evaluate the contribution of each cell type to liver regeneration in live animals. This is unmatched in any other model organism.

Like all experimental models, there are limitations to using zebrafish. As most major advantages of the zebrafish model are associated with the embryonic and larval stage, the majority of zebrafish studies investigating liver regeneration are carried out in young animals. Even though the liver by 5 dpf carry out all functions found in the adult mammalian liver, it is possible that liver regeneration program in young zebrafish has unique properties. For example, recent lineage-tracing experiments in adult mice have failed to observe transdifferentiation of biliary cells into hepatocytes that is readily observed in the embryonic zebrafish liver following severe liver injury (Grompe, 2014). One potential explanation for the observed discrepancies is that embryonic biliary cells are less differentiated and thus harbor more stem-cell like, transdifferentiation potential than adult biliary cells, an important difference that needs to be taken into account for future investigations using the zebrafish embryonic system.

The small body size of zebrafish embryos makes them cost-effective and ideal for large-scale chemical/genetic screens; yet the same attribute makes surgical manipulation highly unfeasible in this model and renders it difficult to directly compare to the mouse partial hepatectomy model for liver regeneration. The recent development of *Tg(fabp10a:mChNTR)* zebrafish may provide an opportunity to bridge this gap. Using partial ablation of hepatocytes which appears to activate a liver regeneration program that parallels the response to total ablation in larvae has suggested that similar programs are activated at both stages (Choi et al., 2014). This indicates that properties governing liver regeneration in larvae may be utilized throughout the zebrafish lifespan.

5. Concluding remarks: How studying liver development and regeneration provides an understanding of liver disease

Zebrafish have historically been used to study embryonic development, and, as a result of this focus for the first few decades when zebrafish were used as a model organism, a majority of studies on the zebrafish liver focused on development. Here, we have reviewed liver development and regeneration in zebrafish and compared the process of regeneration in zebrafish to that in mammals. From these basic studies, several insights have emerged that have informed our understanding of disease. There are extensive recent reviews on the use of zebrafish to study liver disease (Goessling and Sadler, 2015; Grompe, 2014; Wilkins and Pack, 2013b). Here, we focus on the intersection between development and regeneration and focus on biliary disease as an illustrative example of how the basic processes of development have promoted our understanding of liver disease.

Work on biliary disease, in particular, BA, serves as illustrative example of how use of zebrafish combined with our growing knowledge of developmental processes can be utilized to make exciting new discoveries in pathophysiology of liver and biliary disease. We have discussed how zebrafish research on hepatobiliary development has provided a framework to understand how the liver recovers from massive injury. Yet the most common causes of liver disease do not appear to have a developmental origin. Can studies in zebrafish address these, too? The answer is a most decidedly yes. Many labs are actively investigating the basis of diabetes and its impact on the liver, the process of hepatocellular transformation which

generates liver cancer and the effects of hepatocyte injury on the wound healing response that when over-stimulated, leads to liver fibrosis and cirrhosis. In addition, the growing community of toxicology researchers using zebrafish as discussed in chapter 9 of this volume, is leading the charge to expand use of this system to identify causes and cures for toxin-induced liver injury.

The field is rapidly making gains into the fundamental *in vivo* biology of liver disease, as evidenced by the outstanding growth of the Zebrafish Disease Models Society (zdmssociety.org), in particular in the area of liver and other gastroenterological diseases. We look forward to the impact that translational zebrafish studies will make in human disease.

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Figure 1: Zebrafish develop a functional liver within 4 days. Detailed timeline and schematics of the key cellular process driving the differentiation of liver architecture are indicated below the phase-line. Molecular regulators mentioned in the text are listed above the developmental phase they are associated with; for a detailed review of the molecular mechanisms controlling liver development see (Goessling and Stainier, 2016; Wilkins and Pack, 2013b). “hpf” = hours-post-fertilization. “dpf” = days-post-fertilization.

Figure 2:

The liver regenerate itself through three possible mechanisms. Under physiological conditions or mild liver injury, mature hepatocytes proliferate to replace lost hepatocytes. Under severe stress that inhibited or exhausted the proliferative capacity of hepatocyte, progenitor-like cells, which may be derived from the biliary lineage, proliferate and differentiate into mature hepatocytes. “*Fah*^{-/-}” = FAH deficient mice. “*Mdm2*^{-/-}” = MDM2 deficient mice. “PHx” = partial hepatectomy. “PH+AAF” = partial hepatectomy followed by 2-acetylaminofluorene treatment. “CCl₄” = Carbon tetrachloride. “DDC” = 3,5-diethoxycarbonyl-1,4-dihydrocollidine. “CDE” = choline-deficient, ethionine-supplemented. “ANIT” = alpha-naphthyl-isothiocyanate. “NTR + Mtz” = nitroreductase transgenic zebrafish treated with metronidazole.

Figure 3:
Biliary cells as a potential source of hepatocytes in zebrafish with total hepatocyte loss.
Molecular markers used in these studies are listed on the bottom (Choi et al., 2014; He et al., 2014). “Mtz” = metronidazole. “NTR” = nitroreductase. “dpf” = days-post-fertilization.

Table 1.

Transgenic zebrafish provide markers of hepatic cell types.

Cell type	Transgenic marker	Gene marker	References
Hepatocytes	<i>Tg(fabp10a:dsRed)</i> <i>Tg(fabp10a:GFP)</i>	<i>fatty acid binding protein 10a</i>	(Korzh et al., 2001a; Mudumana et al., 2004; Nguyen et al., 2012; Ober et al., 2006) Her et al, 2003
Biliary epithelial cells	<i>Tg(tp1bglob:eGFP)</i> <i>Tg(krt18:eGFP)</i>	<i>notch1</i> <i>keratin 18</i>	Parsons et al, 2009 Wilkins et al, 2014
Stellate cells	<i>Tg(hand2:eGFP)</i>	<i>hand2</i>	Yin et al., 2012
Endothelial cells	<i>Tg(kdrl:mCherry)</i>	<i>kinase insert domain receptor like</i> (also known as <i>flk1</i>)	(Sakaguchi et al., 2008) Wang et al., 2010