Liver Development and Cancer Formation in Zebrafish

Jeng-Wei Lu,1,2 Yu Hsia,1 Hsiao-Chen Tu,1,3 Yung-Chun Hsiao,1,3 Wan-Yu Yang,1 Horng-Dar Wang3 and Chiou-Hwa Yuh1,4,5*

Liver is the largest organ in the human body, and it regulates many physiological processes. Many studies on liver development in different model organisms have demonstrated that the mechanism of hepatogenesis is conserved in vertebrates. The identification of the genes and regulatory pathways involved in liver formation provides a basis for the diagnosis of liver diseases and therapeutic interventions. Hepatocellular carcinoma is the third leading cause of mortality worldwide. In the last decade, genetic alterations, which include the gain and loss of DNA, as well as mutations and epigenomic changes, have been identified as important factors in liver cancer. Many genetic pathways are dysregulated during carcinogenesis. Here, we review the gene regulatory networks that underlie liver organogenesis and the dysregulation of these pathways in liver cancer. The genes and pathways involved in hepatogenesis and liver cancer are largely conserved between zebrafish and humans, making this an ideal model organism for the study of this disease. A better understanding of liver development may aid in the development of new diagnostic and therapeutic approaches to liver cancer. Birth Defects Research (Part C) 93:157–172, 2011. © 2011 Wiley-Liss, Inc.

INTRODUCTION

Liver is an important organ that metabolizes dietary molecules and urea, detoxifies toxic compounds, stores glycogen, and exhibits both endocrine and exocrine properties (Lemaigre and Zaret, 2004; Zorn, 2008; Si-Tayeb et al., 2010). As part of its endocrine functions, the liver secretes many hormones, including insulin-like growth factors, angiotensinogens, and thrombopoietin, as well as serum proteins, such as albumin and apolipoproteins. The liver secretes bile to aid digestion as part of its exocrine function. In addition, the liver contains vasculature in the form of a portal vein, hepatic artery, venuoles, and arterioles, which control blood flow and transport molecules to the circulatory system. The liver also exhibits a regenerative response to injury and an immune response against foreign materials (Si-Tayeb et al., 2010). In the past two decades, scientists have identified the mechanisms by which the liver performs these functions. These complicated tasks are performed by different liver cell types that include hepatocytes, cholangiocytes (bile duct cells), endothelial cells, liver sinusoidal endothelial cells, pit cells (natural killer cells), Kupffer cells (macrophages), and hepatic satellite cells. Although hepatocytes account for 78% of the liver volume, cooperation between different cell types contributes to liver function (Zorn, 2008; Si-Tayeb et al., 2010). The extremely complex liver tissue architecture is crucial for normal liver function. For the past decade, developmental biologists have been studying how the liver differentiates from the endoderm into such a complex organ and how its cells arrange to form its three-dimensional architecture.

During the past decade, scientists have been studying the embryonic development of the liver (hepatogenesis) in the mouse, chick, Xenopus, and zebrafish (Zorn, 2008; Chu and Sadler, 2009; Zorn and Wells, 2009; Si-Tayeb et al., 2010). The collected data on liver development in different model organisms have demonstrated that the mechanism of hepatogenesis is conserved in vertebrates.
knowledge about hepatogenesis is immense, and the results from these studies demonstrate that there are evolutionarily conserved networks that underlie liver development in vertebrates. Although there are still some missing pieces in our understanding of liver organogenesis, animal models have become a promising tool for elucidating the complete gene regulatory networks (GRNs) that direct hepatogenesis.

Because the liver is such a vital organ, liver failure is a life-threatening condition. Liver diseases include hepatic fibrosis, cirrhosis, hepatitis, hepatocellular carcinoma (HCC), and cholangiocarcinoma. HCC is the fifth most common cancer and ranks as the third leading cause of mortality worldwide (Roberts and Gores, 2005; El-Serag and Rudolph, 2007). Many lines of evidence suggest that hepatocarcinogenesis partially recapitulates fetal liver development; both adult cancer cells and fetal liver cells have the capacity for the hallmarks of cancer: self-renewal, sustaining proliferative signaling, enabling replication immortality, resisting cell death, and creating the microenvironment (Kung et al., 2010; Hanahan and Weinberg, 2011). Many differentiated adult HCCs present a less differentiated phenotype than normal liver, and similar to fetal liver supports the progenitor cell differentiation arrest model (Sell and Leffert, 2008). Many signaling pathways, such as the transforming growth factor beta (TGF-β) and Wingless (Wnt)/β-catenin signaling pathways, play important roles in both liver development and HCC (Tang et al., 2008; Ikegami, 2009). In a normal liver, the precise identities and sources of proliferative signals, as well as the mechanisms that control the release of mitogenic signals, remain poorly understood because of their complexity. In contrast, the source of cell survival and proliferative signals during liver development and mitogenic signaling in cancer cells are more fully understood (Lemmon and Schlessinger, 2010; Si-Tayeb et al., 2010; Witsch et al., 2010). Research on liver cell specification, budding, and differentiation during embryogenesis should improve our knowledge and understanding of pathologic liver conditions. Most importantly, a greater understanding of the GRNs involved in liver development should help determine the GRNs of the multiple, distinct cell types present during carcinogenesis.

Zebrafish (Danio rerio) is a popular research model for genetics and developmental biology and is used in a variety of biomedical research fields, including angiogenesis, neurogenesis, organogenesis, human diseases, aging, toxicity, pathology, behavior, cancer studies, and drug screening (Spitsbergen and Kent, 2003; Zon and Peterson, 2005; Lieschke and Currie, 2007). Recently, more advanced technologies as discussed below have been developed and applied in zebrafish research to develop a zebrafish HCC model.

In the first section of this review, the basic mechanisms that control liver organogenesis are summarized, and subjects of special interest (demarcated by subheadings) that illustrate our understanding of these complicated networks follow the first section. In subsequent sections, we address the progress and discoveries made on liver disease and hepatocarcinogenesis over the past decade, with emphasis on the activation of signal transduction pathways during hepatocarcinogenesis (demarcated by subheadings) due to genomic instability and mutations. Finally, recent advances using zebrafish to study the mechanisms of cancer formation and as a drug-screening platform are reported. New techniques that have been developed in zebrafish, which provide both an excellent model for liver disease and a bridge between basic science and translational research, are discussed.

GENE REGULATORY NETWORKS UNDERLIE LIVER DEVELOPMENT

In the past decade, studies of liver development in various model organisms have revealed that an evolutionarily conserved mechanism that includes cell origins, transcription factors, and signaling pathways directs the majority of hepatogenesis. The induction signals from the adjacent mesoderm are also conserved (Zorn and Wells, 2009; Si-Tayeb et al., 2010). With this knowledge, hepatic-like tissue can be induced from embryonic stem cells in vitro using the proper signal ligands (Zorn, 2008).

The Developmental Events and Timeline of Liver Organogenesis in Mice and Zebrafish

Endoderm-derived hepatocytes and cholangiocytes constitute 73% of the liver cell population. The major developmental events of liver organogenesis consist of endoderm formation, hepatic specification, liver bud growth, and hepatocyte/biliary differentiation (left panel of Fig. 1). In mice, liver development proceeds from endoderm patterning during gastrulation and the early somite stages, when differential Wnt and fibroblast growth factor (FGF) signaling is required to induce the formation of the foregut, midgut, and hindgut along the anterior-posterior (A-P) axis. Fate map studies have revealed that the mouse embryonic liver originates from the ventral foregut endoderm at embryonic day 8.0 (e8.0) (Tremblay and Zaret, 2005). The homeodomain factor Hhex becomes enriched in the hepatic endoderm by e8.5 in mice. At e9.5, the hepatic endodermal cells delaminate from the epithelium and invade the septum transversum mesenchyme (STM) to form the liver bud. Between e9.5 and e15, the liver bud undergoes tremendous growth with the help of mesenchymal signals, which include FGF, bone morphogenetic protein (BMP), hepatocyte growth factor (HGF), Wnt, TGF-β, and retinoic acid (Zorn, 2008; Nakamura and Nishina, 2009; Zorn and Wells, 2009; Si-Tayeb et al., 2010).

The zebrafish embryonic fate map at the 50% epiboly stage (Kimmel et al., 1990; Woo and Fraser, 1995; Warga and Nus-
slein-Volhard, 1999; Gritsman et al., 2000; Dougan et al., 2003; Keegan et al., 2004) indicates that the formation of the mesendodermal lineage is established by the end of gastrulation, i.e., ~10 hr postfertilization (hpf) (Kimmel et al., 1995). Multiple signals influence the specification of the endoderm, ectoderm, and mesoderm. Signals that include maternal nuclear β-catenin, BMP, FGF, Nodal, and Wnt all affect cell fates. The endoderm develops from the four most marginal blastoderm tiers of the late blastula-stage embryo under the primary influence of Nodal signaling (Chan et al., 2009). According to the fate map, endodermal cell lineages are primarily derived from the more dorsal and lateral cells of the blastoderm margin (Warga and Nusselein-Volhard, 1999). The endoderm further differentiates into the pharynx, stomach/intestine, and liver. Liver-specific transcription factors, such as hhes and prox1, are initially expressed at 24 hpf, and liver development is complete by 120 hpf.

**Induction Signals and Transcription Factors Essential for Liver Development**

Signals generated by neighboring cells activate signaling pathways and ultimately trigger transcription factors that activate downstream targets. These events further subdivided the territory of the liver into the specialized cell types present in this complicated organ. GRNs control the exact spatial and temporal expression patterns of all genes and the architecture of the system. In Figure 1, the molecular events that occur during hepatogenesis from fertilization to liver maturation are summarized from previous studies.

Many endoderm-specific transcription factors are induced and activated by maternal Nodal and Wnt/β-catenin signals as well as unknown signals emitted from the zebrafish yolk syncytial layer (Chan et al., 2009). Studies on mice, Xenopus, and zebrafish have described a generalized model that shows that a high Nodal level is required to induce the formation of the anterior mesendoderm and endoderm, whereas lower Nodal levels are sufficient for inducing the mesoderm and posterior tissue.

During gastrulation and somite formation, the endoderm cells elongate and become the gut

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**Figure 1.** Gene regulatory networks for hepatogenesis. On the left panel, events for liver formation and the timeline from mouse or zebrafish are indicated. One the right, from top to bottom shows the gene interactions require for each territory highlighted with a different background color. Genes are indicated as lines with arrows, whereas proteins are illustrated as bubbles. Activation of genes is achieved by signal transduction pathways or other genes.
tube. The foregut endoderm receives FGF signals from the neighboring cardiac mesoderm and BMP signals from the STM. Several FGFs (FGF1, FGF2, FGF8, and FGF10) are expressed in the mouse cardiac mesoderm, and knockouts of these FGFs affect liver formation. Although the source of FGF signals in zebrafish is still unclear, the requirement for FGF signaling in hepatic specification is evolutionarily conserved (Si-Tayeb et al., 2010). In mice, BMP4 is under the control of Gata4 and is expressed in the STM to regulate early hepatic development; however, other BMP family members, such as BMP2, are also present in the immediate vicinity. The requirement of BMP signals for the induction of hepatic specification is conserved in zebrafish; nevertheless, the source of the BMP signal originates in the lateral plate mesoderm rather than the STM. The Wnt antagonist (sFRP5) is required to release Wnt repression of Hhex expression in the anterior endoderm at the hepatic specification stage. Subsequently, the Wnt signal (wnt2bb) expressed in the lateral plate mesoderm is essential for the onset of the differentiation of hepatic progenitor cells in zebrafish, and the requirement for Wnt in promoting hepatogenesis is evolutionarily conserved (Si-Tayeb et al., 2010).

Many evolutionarily conserved transcription factors are expressed in the anterior endoderm, including gata4, gata6, foxa1, foxa2, hnf1b, prox1, and hhex. An analysis of Hhex knockout mouse embryos found that Hhex is required for early bud morphogenesis and differentiation into hepatocytes (Keng et al., 2000; Hunter et al., 2007). Previous studies in mice have shown that GATA4 and GATA6 may positively regulate the expression of Hhex by binding to its promoter (Denson et al., 2000; Zhao et al., 2005). In zebrafish, hhex is expressed in the hepatic bud from 22 to 50 hpf, and knockdowns of hhex with morpholinos perturbed liver development in a dose-dependent manner (Wallace et al., 2001). Overexpression of the dominant-negative FGF receptor between 18 and 26 hpf has been shown to decrease the expression of gata4, gata6, prox1, and ceruloplasmin in zebrafish (Shin et al., 2007). Mutations in lost-a-fin or expression of a dominant negative form of the BMP/activin receptor has been shown to result in reduced expression of hhex and prox1 in the liver regions of zebrafish embryos (Shin et al., 2007).

Following the specification stage, the liver bud undergoes tremendous growth in all of the species in vertebrate. The liver then becomes the major site of fetal hematopoiesis in mice; however, the hepatic vasculature and hematopoiesis are not essential for zebrafish liver development. Many ligands are expressed in the surrounding mesoderm tissue (STM and hepatic mesenchyme in mice), including BMP, FGF (via PI3 kinase), HGF, Wnt, TGF-β, and retinoic acid. The receptors and components downstream of the signaling pathways, including BMPR, FGF, c-MET (tyrosine kinase receptor of HGF), TGF, PI3K, JNK, Arf6, Raf1, Smad2/3, β-catenin, c-Jun, Tbx3, and NF-κB, are expressed in hepatocytes (Zorn, 2008). Tumor necrosis factor-α (TNF-α) binds to TNFR and activates three separate signaling pathways: the cell death, cell survival, and cell proliferation signals (Nakamura and Nishina, 2009). In promoting cell proliferation, TNF-α/TNFR1 and HGF/c-MET can both activate MKK4 and MKK7, which then activate JNK; JNK activation promotes c-JUN phosphorylation and cdc2 gene expression (Nakamura and Nishina, 2009). Simultaneously, TNF-α/TNFR1 activates NF-κB signaling for cell survival that contradicts the cell death signal.

In mouse embryos, bipotential hepatoblasts differentiate into hepatocytes or biliary epithelial cells (BECs) on approximately day e13. The hepatoblasts that are surrounded by the portal mesenchyme become BECs in response to the TGF-β and Wnt signals sent from the portal mesenchyme. These signals downregulate the expression of prohepatic transcription factors, such as NHF4α, Tbx3, and C/ebp. These mesenchyme signals also upregulate the expression of biliary epithelial cell-specific transcription factors, such as Onecut1 (Oc1), Onecut2 (Oc2), hhex, and hnf1b. In addition, foxm1b mutant embryos lack BECs. Continuous signals (Notch, epidermal growth factor (EGF), and HGF) from the portal mesenchyme are essential for ductal plate remodeling, whereas other signals (OSM, Dex, HGF, and Wnt) promote hepatocyte maturation (Zorn, 2008). Figure 1 illustrates the GRNs that direct liver organogenesis from endoderm specification to hepatocyte/biliary differentiation. There are many missing links in this representation, such as the intramodular links, and the direct binding; among these transcription factors and signaling pathways is not well elucidated and requires further study.

**Epigenetic Changes Associated with Liver Development in Zebrafish**

Epigenetic regulation of gene expression also plays an important role in liver development in zebrafish. The two prevailing forms of epigenetic control over gene expression are DNA methylation and histone acetylation (Chu and Sadler, 2009; Hamilton, 2010). Transcriptional inactivation can be achieved via DNA methyltransferase (DNMT), which adds a methyl group at the 5' position of a cytosine base, or histone deacetylases (HDACs), which remove an acetyl group from an ε-N-acetyl lysine amino acid on a histone (Okano et al., 1999; Bird, 2002; Choudhary et al., 2009). Analysis of dnmnt or hdac mutant embryos has demonstrated that epigenetic mechanisms control both hepatic specification and liver bud outgrowth in zebrafish.

In zebrafish, the knockout of both hdac1 and hdac3 has been found to result in severe embryonic development defects, in which aberrant hdac3 produced
greater effects on the liver than hdac1; hdac1 depletion affected liver size and caused the formation of ectopic endocrine tissue (Faroq et al., 2008; Noel et al., 2008). Dnmt1 mutant embryos undergo normal hepatic patterning but differentiate with increased apoptosis; therefore, the liver cannot grow larger. The uhrf1 gene is essential for maintaining DNA methylation via the recruitment of Dnmt1 to hemimethylated DNA in mammalian cells. Abnormalities in either of these genes contribute to epigenetic changes that cause hepatocytes to undergo apoptosis (Bostick et al., 2007; Sharif et al., 2007; Chu and Sadler, 2009). The results summarized above indicate that epigenetic changes significantly influence liver development; however, this represents a new avenue of liver development research.

LIVER DISEASE AND HEPATOCARCINOGENESIS

Because the liver is such a vital organ, liver failure is a life-threatening condition. Many etiological factors, such as chronic exposure to aflatoxin B1, alcohol consumption, and chronic viral infection with the hepatitis B virus (HBV) or hepatitis C virus (HCV), can cause liver damage (Morgan et al., 2004; McGlynn and London, 2005; Seeff and Hoofnagle, 2006; El-Serag and Rudolph, 2007; Marrero and Marrero, 2007). The development of HCC involves multiple steps that include steatosis, fibrosis, cirrhosis, adenoma, and carcinoma (Tarantino et al., 2007). In fact, it is believed that more than 80% of all HCC cases are the result of infection by either HBV or HCV (Chen et al., 1997). Two billion people worldwide have been infected with HBV; of these cases, 360 million suffer from chronic infection, and 600,000 die each year from HBV-related liver disease or HCC (Shepard et al., 2006). HCC is one of the deadliest cancers, and there is still no effective therapy available. Thus, an understanding of the molecular mechanisms involved in hepatocarcinogenesis and the development of therapeutic approaches to treating liver cancer have become important.

GENETIC ALTERATIONS IN HEPATOCELLULAR CARCINOMA

The pathogenesis of HBV-associated HCC has been extensively studied, and molecular changes that occur during malignant transformation have been identified. It has been postulated that the insertion of HBV DNA into the human genome results in chromosomal instability that causes cancer formation by several different mechanisms. Chronic HBV infection may trigger specific oncogenic pathways and cause the accumulation of genetic and epigenetic alterations in regulatory genes (Cougot et al., 2005; Tsai and Chung, 2010) that promote HCC. Transactivation of oncogenes, inactivation of tumor suppressor genes (TSGs), and alteration of the cell cycle by HBV proteins are all involved in the progression of hepatocellular carcinogenesis.

Hepatocarcinogenesis is a multistep process that involves genetic alterations, including gain or loss of DNA, mutation of oncogenes and tumor suppressors, dysregulation of signaling pathways, epigenomic changes, and changes in the expression of microRNA (Hoshida et al., 2010; Zucman-Rossi, 2010). The development of new high-throughput genomic technologies has promoted the classification of the molecular diversity in human liver cancer and allowed us to understand the multiple steps of hepatocarcinogenesis (Hoshida et al., 2009, 2010; Ung et al., 2009).

Analysis of 137 tumors using high-density allelotyping revealed that a β-catenin mutation associated with chromosome 8p losses was related to a chromosome stability group (Laurent-Puig et al., 2007). Similarly, analysis of 60 tumors discovered that β-catenin mutations were associated with a chromosome stability group. However, losses in chromosome 8q were not found in this study (Zucman-Rossi, 2010). In addition, an HNF1A mutation, CDH1 methylation, and Wnt pathway activation were associated with a genomic stability group during hepatocarcinogenesis (Boyard et al., 2007). In a chromosome instability group, many of the chromosome areas exhibited the most frequent allelic losses, and axis inhibition protein 1 (AXIN1) and p53 were frequently mutated (Laurent-Puig et al., 2001). Similarly, analysis of 60 tumors found that AXIN1 and p53 mutations were associated with a chromosome instability group (Boyard et al., 2007). Some chromosomal loss of heterozygosity (LOH) is associated with a chromosome instability group of the HCC that includes 4q, 13q, 16p, 16q, and 17p from both studies; other genomic losses were found in one study but not in the other. These chromosomal regions contain key players in HCC, such as p53 (chromosome 17p), Rb (chromosome 13q), AXIN1, and cyclin-dependent kinase inhibitor 2A (CDKN2A) (Laurent-Puig and Zucman-Rossi, 2006). Other genetic alterations in the genomic instability group include mutations in PI3K2CA and the methylation of CDKN2A, as well as the activation of the mitotic cell cycle, the AKT pathway, and developmental and imprinting genes, e.g., insulin-like growth factor 2 (IGF-2) (Boyard et al., 2007; Zucman-Rossi, 2010). The signatures of 16 genes to classify the HCC would be clinically useful for determining the dysregulated pathways and predicting drug response (Boyard et al., 2007). Here, we have summarized the most important genetic alterations in human HCC. Table 1 lists all of the signaling pathways and downstream cascades responsible for hepatogenesis and hepatocarcinogenesis.

Alterations of the Wnt/β-Catenin Signaling Pathway

Inappropriate reactivation of the Wnt pathway that results from
alterations in the β-catenin gene (CTNNB1) has been implicated in liver oncogenesis (Buendia, 2000). β-Catenin is the most frequently observed mutation-activating oncogene in HCC; alterations in this gene are found in 20 to 50% of HCC patients (de La Coste et al., 1998; Miyoshi et al., 1998).

β-Catenin has dual functions in adhesion and Wnt signaling. N-terminal mutations of β-catenin trigger dominant oncogenic activity (Morin et al., 1997), and the loss of consensus phosphorylation sites on β-catenin has been identified in many mutations, which suggests that β-catenin is negatively regulated by GSK3β/APC/axin via phosphorylation (Laurent-Puig and Zucman-Rossi, 2006). The mutation rates of the tumor suppressors AXIN1 and AXIN2 in human HCC are ~5 to 25% and 3 to 10%, respectively (Ishizaki et al., 2004; Zucman-Rossi et al., 2007; Zucman-Rossi, 2010).

A previous study that investigated HCC cell lines, HB (hepatoblastoma), and primary HCC showed that mutations in the CTNNB1, AXIN1, and AXIN2 genes caused Wnt/β-catenin pathway dysregulation (Taniguchi et al., 2002). All of the CTNNB1 mutations in HCC were missense mutations, minor deletions, or small insertions (Jeng et al., 2000). The AXIN1 mutations included truncation mutations due to either small deletions or nonsense mutations, which encoded a truncated protein in the cytoplasmic GSK3β complex that inhibited the Wnt pathway; this suggests that AXIN1 functions as a tumor suppressor. Inactivation of AXIN1 prevents phosphorylation of β-catenin by GSK3β, which leads to an accumulation of β-catenin and the activation of Wnt target genes (Laurent-Puig and Zucman-Rossi, 2006). Previous studies have shown that AXIN2 is a transcriptional target of the TCF/LEF transcription factor complex downstream of activated β-catenin (Jho et al., 2002). AXIN2, which is mutated in 3 to 10% of HCC cases, functions as an antagonist by promoting β-catenin degradation (Ishizaki et al., 2004; Zucman-Rossi, 2010). These results may explain the presence of tumors with mutations in both the CTNNB1 and AXIN1 or AXIN2 genes, which contribute to the activation of the Wnt signaling pathway (Taniguchi et al., 2002).

In the canonical Wnt signaling pathway, Wnt binds to its cell surface receptor, causing dissociation of β-catenin from the APC complex and preventing degradation (Chu and Sadler, 2009). Wnt/β-catenin signaling is activated relatively early during development and regeneration. When activated, Wnt/β-catenin signaling switches on the expression of target genes. These downstream target genes are important in cell cycle progression and contribute to the initiation of the regeneration process (Nejak-Bowen and Monga, 2010). Dysregulation of Wnt/β-catenin signaling was found in zebrafish liver tumors (Lam et al., 2006); dysregulation of ctnnb, wif1, wnt2, ctnnbip1, and ccnd1 also suggested the presence of deregulation in the Wnt/β-catenin signaling pathway in zebrafish liver tumors (Lam and Gong, 2006).

Alterations in the p53 Gene

The most frequently mutated TSG in HCC is p53 (Hsu et al., 1991; Zucman-Rossi, 2010), which is activated in response to DNA damage and either promotes apoptosis or induces cell cycle arrest to permit DNA repair (Levine et al., 1991). The p53 TSG is located on chromosome 17p13.1 (Isobe et al., 1986) and plays a major role in HCC, irrespective of the etiology (Edamoto et al., 2003). In more than 50% of HCC tumors, a G → T transversion at codon 249 of p53 was found after high aflatoxin B1 (AFB1) exposure (Bressac et al., 1991). In contrast, patients who were not exposed to AFB1 had lower rates of p53 gene mutation (20%) without specific codon hotspots (Laurent-Puig and Zucman-Rossi, 2006). Moreover, analysis of intra-tumoral nodular lesions within HCC samples has found genetic heterogeneity in p53, and the p53 mutation has been proposed to correlate with shortened survival and a poor prognosis (Honda et al., 1998; Buendia, 2000).

Alterations in the Retinoblastoma Protein, CDKN2A, and Gankyrin

The tumor suppressor retinoblastoma protein (Rb) is critical for the development of several cancer types. In normal cell signaling, Rb prevents cell division and cell cycle progression. Frequent allelic deletions on chromosome 13q that cause inactivation of the tumor-suppressor Rb gene located at 13q14 have been observed in HCCs (Friend et al., 1986). LOH at the Rb locus has been observed in 25 to 48% of cases of HCC (Kuroki et al., 1995), and pRb expression has been shown to be strongly downregulated in 30 to 50% of tumors, which correlates with genetic alterations in the p53 gene (Buendia, 2000). However, an Rb mutation alone is found in less than 11% of HCC cases (Zhang et al., 1994). This result is indicative of the heterogeneity of human HCC.

There are many different ways of inactivating pRb, including deleterious mutations in the gene itself, loss of TGF-β responsiveness, and the inactivation of cyclin D-dependent kinase inhibitor 2A (CDKN2A). CDKN2A functions as a tumor suppressor in the retinoblastoma pathway (Hickman et al., 2002), and it is mutated in 10 to 60% of HCC patients (Zucman-Rossi, 2010). In HCC, LOH at 9p occurs in ~20% of cases, and homozygous deletions at 9p21 (where CDKN2A is located) have been detected (Liew et al., 1999). In the majority of tumors, inactivation of the gene was achieved by de novo methylation of the CDKN2A promoter, which led to the absence of protein expression in 30 to 70% of cases (Matsuda et al., 1999).

Overexpression of gankyrin, an oncoprotein that contains seven ankyrin repeats, has been found in HCC patients (Higashitsuji et al., 2000). Gankyrin downregulates p53 protein levels via ubiquityla-
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<td>gata6, gata4, hnf1b, foxa1, foxa2, prox1, and hhexβ1-integrin</td>
<td>1. Specification of three germ layers 2. Mesenchymal signals for hepatic specification 3. Liver bud growth</td>
<td>1. BMPs overexpression 2. TGF-β signal pathway activated by HBx</td>
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<td>1. Specification of three germ layers 2. Inhibited at hepatic specification stage 3. Promoting liver bud growth 4. Promoting hepatocyte maturation</td>
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<td>Ras/Raf/ MAP2K/MAPK</td>
<td>id3</td>
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<td>1. Ras mutations 2. Raf hyperactivated 3. MEK1/2 overexpression 4. ERK1/2 overexpression 5. EGF overexpression</td>
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<td>EGF, PDGF, and VEGF</td>
<td>EGFR, PDGFR, and VEGFR</td>
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**TABLE 1.** Signaling Pathways and Downstream Cascades Responsible for Hepatogenesis and Hepatocarcinogenesis
tion and degradation (Kim et al., 2009). Gankyrin also binds to Rb, accelerating the degradation of Rb in vivo and in vitro (Higashitsuji et al., 2000). Collectively, frequent alterations in Rb, CDKN2A, and gankyrin play an important role in hepatocarcinogenesis.

**DYSREGULATED SIGNALING PATHWAYS IN HEPATOCARCINOGENESIS**

Previous studies have reported the occurrence of aberrant activation of signaling pathways to sustain proliferative signaling (e.g., the EGF and RAS/mitogen-activated protein kinase pathways), to resist cell death (e.g., Akt, the mechanistic target of the rapamycin pathway), to enable replicative immortality (e.g., the Wnt and Hedgehog pathways), and to induce angiogenesis (e.g., vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) pathways), which are capabilities acquired during the multistep development of human tumors (Hoshida et al., 2010). The first fundamental hallmark of cancer is its ability to sustain chronic proliferation. In the past decade, many signaling pathways have been found to be dysregulated in cancer, contributing to tumor formation and progression. These pathways include the Wnt/β-catenin (as described earlier), HGF/c-MET, EGFR, IGF, MAPK, and PI3K/akt/mTOR pathways (Nejak-Bowen and Monga, 2010; Whittaker et al., 2010; Zender et al., 2010). These pathways play significant roles in liver organogenesis. Human and zebrafish liver tumors share a molecular framework that is deregulated during tumorogenesis, which is indicative of the evolutionarily conserved properties of these pathways and their downstream transducers (Lam and Gong, 2006; Lam et al., 2006).

**The HGF/c-MET, EGFR, and IGF Signaling Pathway**

c-MET, the HGF tyrosine kinase receptor, is predominantly expressed on the surface of epithelial and endothelial cells. The HGF ligand exerts its effects by binding to c-MET, which regulates many important events during embryogenesis, including cell proliferation, migration, survival, branching morphogenesis, and angiogenesis. Upon HGF binding to c-MET, the signal cascade occurs via phosphorylation of the adaptor proteins growth factor receptor-bound protein 2 (GRB2) and GRB2-associated-binding protein 1 (GAB1), which then activate downstream effectors, such as phospholipase C, PI3K, and ERK (Whittaker et al., 2010).

The receptor for IGF1, IGF1R, is a key regulator of anchorage-independent growth (Whittaker et al., 2010). Following liver damage or viral transactivation, the IGF-2 receptor is upregulated by altered methylation of the IGF-2 promoter (Feitelson et al., 2004; Whittaker et al., 2010). In zebrafish HCC, several IGF-binding proteins (IGFBPs), such as igfbp2b, were significantly hypomethylated and may have upregulated the expression of IGF-2 (Mirbahai et al., 2011). In the early stages of tumorogenesis in highly proliferating tumor cells, the lack of a vascular supply results in hypoxia (Kelly et al., 2008; Mirbahai et al., 2011). The anaerobic conditions and the presence of IGF result in increased expression of hypoxia-inducible factor 1 (HIF-1) (Kelly et al., 2008).

**The MAPK Pathway**

The mitogen-activated protein kinase (MAPK) pathways regulate crucial cellular processes during development, including proliferation, differentiation, angiogenesis, and survival (Whittaker et al., 2010). The MAPK signaling pathways play vital roles in embryogenesis and are often deregulated in various types of human cancer, including HCC. There are at least four subfamilies of MAPKs: extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs), p38 MAPKs, and ERK5. The activation of the Ras-MAPK pathway in zebrafish liver tumors is indicated by upregulation of shc1, mapk1, dusp4, dusp6, and genes associated with Ras GTPases, e.g., rhoc, rhogap1, cdc42, rac1, g3bp2, and gef10 (Lam and Gong, 2006). Constitutive activation of a MAPK pathway has been found in ~40% of melanoma patients due to mutations of the B-Raf protein (Davies and Samuels, 2010).

**The PI3K/AKT/mTOR Signaling Pathway**

In normal tissue, the PI3K/AKT/mTOR signaling pathway targets the lipid products of PI3K for dephosphorylation. The PI3K/AKT/mTOR pathway is negatively regulated by the tumor suppressor phosphatase and tensin homolog (PTEN) (Roberts and Gores, 2005; Whittaker et al., 2010). Binding of IGFs or EGF to their receptors activates PI3K (Avila et al., 2006; Whittaker et al., 2010) and the downstream signal pathway. The PTEN gene is mutated in 5 to 10% of human HCC cases (Bamford et al., 2004; Whittaker et al., 2010), which results in the constitutive activation of the PI3K/AKT/mTOR pathway (Hu et al., 2003; Whittaker et al., 2010). The PI3K/AKT/mTOR pathway plays a critical role in the pathogenesis of HCC (Whittaker et al., 2010).

**Epigenomic Changes Associated with HCC**

Several other genes from families that include ABCA, CHST, DDX, KCTD, MEGF, MYO, NPY, RNF, and TBCID have been found to be hypermethylated in both zebrafish and human HCCs. The genes with altered methylation in zebrafish HCC are associated with biological functions, such as cell death, cell morphology, inflammatory response, DNA repair, and replication, and induced molecules involved in cancer formation, such as c-jun, shc, and pka (Mirbahai et al., 2010). Many HCCs exhibit methylation of at least one TSG promoter, particularly SGS-1, APC, E-cadherin, and p15. Several epigenetically
silenced or aberrantly methylated putative TSGs found in HCCs were also inactivated in the non-tumorous liver. However, the frequency of methylation in tumorous liver is much higher than in nontumorous liver. Moreover, methylation is observed more frequently in HCV carriers than in HBV/HCV-double-negative HCC cases (Yang et al., 2003; Calvisi et al., 2007).

Hepatocarcinogenesis is associated with increased levels of DNMT1, DNMT3a, and DNMT3b mRNA and a progressive increase in the number of epigenetically silenced genes compared to normal liver and chronic hepatitis-, cirrhosis-, and HCC-affected livers (Oh et al., 2007). These combined epigenetic events facilitate the estimation of prognosis and risk of recurrence of HCC. Epigenetic changes could be used to customize therapy and predict future survival.

HEPATOCARCINOGENESIS RELATED TO THE HBV X ANTIGEN

One of the proteins encoded by HBV, the hepatitis B virus X protein (HBx), caused enhanced colony formation or transformation of cells in vitro in various cell lines (Koike et al., 1989; Shirakata et al., 1989; Seifer et al., 1991; Zhang et al., 2009). Several HBx transgenic mouse models that develop HCC have also been created (Kim et al., 1991; Ullrich et al., 1994; Wu et al., 2006). A transgenic mouse model, in which the albumin promoter drives the expression of HBx, has been shown to develop HCC at 14 to 16 months of age without chemical treatment (Wu et al., 2006, 2008). We have used this mouse model to identify biomarkers for the early stages of HCC formation. Here, we have summarized the effect of HBx on carcinogenesis (Figure 2).

HBx Enhances Genomic Instability

As in most cancers, gross chromosomal abnormalities have been credited with contributing to cellular transformation and tumor progression in HBV-associated HCC (Gatza et al., 2005). Genomic instability has been explicitly linked to the expression of HBx. HBx increases the levels of several cellular oncoproteins through the amplification of chromosome regions that contain oncogenes and the deletion of genomic regions that encode tumor suppressor genes, cause HCC to become metastatic and incurable.
HBx (Slagle et al., 1996). The rate of chromosomal alterations is significantly increased in HBV-related tumors compared to tumors associated with other risk factors. HBV may therefore play a role in enhancing genomic instability (Cougot et al., 2005).

**Signal Transduction Pathways Affected by HBx**

HBx may contribute to the development of HCC via the activation of signaling pathways, such as NF-κB; these pathways affect TGF-β1 expression. This contribution was demonstrated by comparing HBx-positive to HBx-negative HepG2 cells (Pan et al., 2004). HBx can also shift TGF-β signaling from the tumor-suppressive to the oncogenic pathway in the early carcinogenic process by the activation of JNK kinase, which phosphorylates Smad3 at a linker region instead of the C-terminus (Murata et al., 2009). TGF-β participates in many different stages of liver organogenesis, from endoderm formation to hepatic specification and liver bud growth (Zorn and Wells, 2009). In the fetal liver, TNF-α engages TNF receptor 1 (TNFR1) and activates NF-κB signaling to transmit the cell survival signal (Nakamura and Nishina, 2009).

HBx may upregulate the expression of multidrug resistance protein (MDR1) and inhibit apoptosis through the activation of the Src and PI3 kinase pathways (Kang-Park et al., 2006). HBx acts as a tumor inducer and stimulates the neoplastic transformation of normal cells, but HBx shifts its function to induce apoptosis in association with Ras (Wei et al., 2006). HBx can be phosphorylated by ERK, and the phosphorylated form has been found to repress the transcription of p21(WAF1/Cip1) and to translocate from the cytoplasm to the nucleus (Noh et al., 2004). HBx also induced centrosome hyperamplification and mitotic aberration via the activation of the Ras-MEK-MAPK pathway. These findings may provide a possible mechanism by which HBx causes genomic instability in an HBV-infected liver (Yun et al., 2004). The protein kinase C pathway (Luber et al., 1993), RAS/RAF/MAP kinase cascade (Benn and Schneider, 1994), c-Jun N-terminal activating protein kinase (Benn et al., 1996), and JAK1/STAT pathways have been found to be subject to activation by HBx (Lee and Yun, 1998). Based on these studies, HBx interacts with many signal transduction pathways to induce HCC, and HBx may have different functions that depend on its association with different transduction pathways at early or late stages of viral infection.

**Transactivation of Cellular Genes**

HBx has been found to increase the levels of fibronectin (FN) mRNA and protein via the HBx-mediated transactivation of the FN promoter, which is NF-κB dependent. HBx also antagonized the repression of the FN promoter by the p53 tumor suppressor. Hence, the FN gene may be a natural target for HBx transactivation, perhaps through the activation of NF-κB and the inactivation of p53, thereby contributing to the accumulation of FN in the liver over the course of chronic HBV infection (Norton et al., 2004). It has been reported that HBx can activate all of the Pol I, Pol II, and Pol III genes via activation of expression of the TATA-binding protein, which is a component of the basal transcription apparatus (Wang et al., 1995).

**Physical Binding and Functional Inactivation of the p53 Cellular Tumor Suppressor Protein**

Tumor development precisely correlates with p53 binding to HBx in the cytoplasm and the complete blockage of p53 entry into the nucleus (Ueda et al., 1995). An analysis of tumor cell DNA showed no evidence of p53 mutation except in advanced tumors, where a small proportion of cells may have acquired specific base substitutions. These results suggest that genetic changes in p53 are late events that may contribute to tumor progression (Ueda et al., 1995). In addition to aberrations in the p53 gene, loss of the Rb gene or LOH at chromosome 13q was observed in six of seven informative cases of eight tumors that carried a mutated p53 gene (Murakami et al., 1991).

**Epigenomic Changes Associated with HBx**

HBx has been reported to repress E-cadherin expression via activation of the DNA methyltransferase-mediated hypermethylation of the E-cadherin promoter (Lee et al., 2005). In a separate study, glutathione S-transferases P1 (GSTP1), enzymes that defend cells against damage mediated by oxidants and electrophilic carcinogens, were suppressed through the hypermethylation of their promoter regions. These data indicate that epigenetic silencing of GSTP1 gene expression via CpG island DNA hypermethylation is common in human HBV-associated HCC (Zhong et al., 2002).

**A ZEBRAFISH ANIMAL MODEL FOR THE STUDY OF LIVER DISEASES AND HCC**

Although the main focus of zebrafish research has generally been on developmental biology, laboratory observations of zebrafish have resulted in the identification of diseases that are similar to those found in humans, such as cancer. Thus, zebrafish became an animal model for human disease, and dozens of studies that used zebrafish as a cancer model have been published in the last decade (Feitsma and Cuppen, 2008).

Increasing rates of HCV infection have been associated with an increase in the incidence of HCC in the United States (El-Serag et al., 2003). HCV-induced hepatocarcinogenesis is widely reported to be due to the HCV core protein, which inhibits p21 expression through inhibition of the TGF-β pathway (Lee et al., 2002). In a transgenic zebrafish model, the HCV core protein induced HCC with or without treatment with thioacetamide, which is a hepatotoxin. However,
thioacetamide treatment can accelerate HCC development by twofold to yield fully developed HCC in 6 weeks (Rekha et al., 2008).

As previously mentioned, endemic HBV infection is strongly correlated with the high prevalence of HCC in Asian countries (Parkin et al., 2001). Moreover, the HBx viral protein has been shown to modulate cell proliferation and induce HCC. In a transgenic zebrafish model, HBx under the control of a liver-specific promoter resulted in hepatic fat accumulation during the progression of hepatitis. Transgenic fish that express viral genes could be an excellent animal model for HCC for studying accelerated cancer formation, induction of fatty liver, and the synergistic effects of different risk factors, including hepatotoxin.

One of the advantages of using zebrafish as a high-throughput screening method for carcinogens is the ease of manipulation. Most of the carcinogens added to embryos have induced neoplasms derived from many tissues, such as epithelial, mesenchymal, neural, and neural crest. The liver is a primary target organ for most carcinogens, regardless of the developmental stage of the fish at exposure. Low ppb concentrations of AFB1 are usually used for dietary carcinogenesis studies in rainbow trout to produce a high incidence of liver neoplasia (Bailey et al., 1996).

Zebrafish larvae are also an attractive model for studying alcoholic liver disease (ALD). In humans, acute alcohol abuse can result in steatosis, which may progress to more severe hepatic disease. The alcohol metabolism pathways in zebrafish are similar to those in humans, and the zebrafish liver is mature in larvae by 4 days postfertilization (dpf). Moreover, zebrafish larvae develop steatosis, which is a sign of ALD, as a result of alcohol being added to the water. The activation of Srebp is required for steatosis in the zebrafish ALD model. Deciphering the molecular pathogenesis of the zebrafish ALD model became possible following the almost complete sequencing of its genome and by using a genetics tool (Passeri et al., 2009).

Several zebrafish strains have become powerful models for elucidating the mechanisms of carcinogenesis and are superior in vivo systems for rapid screening of anticancer genetic or chemical factors (Rekha et al., 2008). In conclusion, the zebrafish is an excellent model to delineate the mechanisms that underlie hepatocarcinogenesis and as a therapeutic drug-screening platform.

New Transgenic Technology for Studying HCC Using Zebrafish

Genetic screening that identifies genes required for developmental processes has been successfully performed in zebrafish. Previously, forward genetic screening in zebrafish identified mutants that developed hepatomegaly, which is a symptom of many liver disorders. Several new genes that play important roles in liver development, physiology, and pathology have been identified using forward genetic screening (Sadler et al., 2005).

The transgenic technologies available in zebrafish have improved over the last 2 decades (Stuart et al., 1988, 1990). Different delivery systems for the transgene, such as the injection of linear DNA (Stuart et al., 1988), supercoiled plasmid DNA (Stuart et al., 1990; Culp et al., 1991), or recombinant bacterial artificial chromosomes into early-stage embryos (Culp et al., 1991) have been developed in zebrafish. Recently, a new transgenic technology, Tol2-mediated transgenesis, has been established. The Tol2 element is a naturally arising, active transposable element discovered in fish genomes. The Tol2 transposon system is considered to be a useful gene transfer vector in vertebrates ranging from fish to mammals (Urasaki et al., 2006). Using coinjection of in vitro-transcribed Tol2 RNA, the DNA fragment surrounded by the Tol2 element transposon can be efficiently excised and integrated into the genome (Kawakami et al., 2000). Tol2-mediated transgenesis is an excellent method for creating transgenic zebrafish because of the high transposition efficiency and the capacity to transfer a large DNA fragment (Urasaki et al., 2006).

Initially, many zebrafish laboratories created green fluorescent protein (GFP) reporter transgenic lines as a marker for cells expressing the gene of interest. Recently, as transgenesis has become common in zebrafish laboratories, researchers have tested mammalian promoters, promoters from other fish species, and endogenous tissue-specific promoters to drive transgene expression (Deiters and Yoder, 2006). One of the most useful GFP transgenic fish lines came from a liver-specific promoter, liver fatty acid-binding protein (L-FABP) (Andre et al., 2000; Denovan-Wright et al., 2000). In the liver, L-FABP plays an important role in the intracellular binding and trafficking of long-chain fatty acids. Isolation of the zebrafish L-FABP promoter and construction of GFP fish lines have been previously performed (Her et al., 2003a,b). Oncogenes driven by the L-FABP promoter have become a useful system for studying HCC in the zebrafish model.

Studying Metastasis in Zebrafish Using the Xenotransplantation Method

The zebrafish is a vertebrate with a complex circulatory system and genetics that are similar to humans, which makes related experiments feasible (Weinstein, 2002). The zebrafish is an excellent model for cancer research. There are many advantages of the zebrafish compared to the mouse (Lam et al., 2006), including ease of experimental handling, drug treatment, and high-throughput screening, as well as the optical transparency of the vascular system and the feasibility of forward and reverse genetic approaches (Thiss and Zon, 2002). Recent
studies have demonstrated the possibility of injecting human cancer cell lines into zebrafish (Weinstein, 2002). In these studies, 2-day-old embryos were injected with 50 to 1000 cells in the yolk sac or near the vascular system. However, in the blastula stage, 1 to 100 cells were sufficient for tumor mass engraftment (Nicoli et al., 2007). Studies of the recipient animals posttransplant are critical for determining tumor engraftment or metastasis (Taylor and Zon, 2009). In a successful xenotransplantation, the migration of CM-DII or cancer cells labeled with another florescent dye can be traced in living embryos (Marques et al., 2009). Tumor angiogenesis induced by the cancer cells can also be investigated in an embryo 3 dpf. Cell invasion and angiogenesis are dynamic processes; compared to a fluorescent microscope, a high-resolution confocal microscope can provide high quality, dynamic, and three-dimensional section images (Stoletov et al., 2007). When mice are used as a tumor transplantation model, only two to three animals can be created for one experiment; in contrast, many more zebrafish than mouse embryos can be injected. In published transgenic line experiments, more than 500 one-cell-stage embryos were injected in a single day, generating 100 transplanted embryos, which is more prolific than the mouse model (Taylor and Zon, 2009). Currently, the tumor transplantation assay is a popular and much easier method to test carcinogenesis and screen cancer stem cells (White et al., 2008). In conclusion, zebrafish represents a promising animal model for tumor xenotransplantation and carcinogenesis research.

CONCLUSION

The basic mechanisms that control liver formation are presented in the first section of this review. Evolutionarily conserved GRNs that direct hepatogenesis are described. Many signaling pathways and genes are activated during the developmental process. Although there are many reviews on liver organogenesis and development, to our knowledge, ours is the first review in which the relationships between different events have been connected within the network architecture at the molecular level.

In the second section of this review, we have summarized known pathological liver conditions, primarily focusing on HBx-induced genomic instability and activation of signaling pathways and transcription as well as epigenetic status. The pathways that are deregulated in HCC are also discussed. There are common features shared by embryonic liver development and liver carcinogenesis. The signaling pathways, transcription factors, and molecular machinery that dictate these events are used in both situations; however, mutations and changes at the genomic or epigenomic level, respectively, occur in the case of tumorgenesis. Although we have a good understanding of organogenesis, establishing how GRNs underlie hepatocarcinogenesis remains a challenge.

Lastly, we have highlighted here examples of exciting research that have utilized zebrafish as a human disease model, especially for liver cancer. The zebrafish has been used by developmental biologists to decode developmental GRNs, and cancer biologists have used the zebrafish to create models for liver cancer via transgenesis and xenotransplantation. The most significant advantages of using zebrafish in cancer studies are the low cost of high-throughput drug screening and the ease of toxicity screening. Zebrafish thus constitutes a bridge between basic science (i.e., liver development) and translational research (i.e., liver cancer).

FUTURE PERSPECTIVES

The study of liver development has demonstrated that many transcription factors and signaling pathways and their components, as well as epigenetic changes, are essential for the specification, growth, and differentiation of the liver. Gene mutations, gains or losses of DNA, and changes in methylation status of transcription factors and signaling pathways contribute to liver disorders and cancer formation. With the help of the zebrafish model and new technologies, deciphering the GRNs that underlie hepatocarcinogenesis and finding a cure for liver cancer are possible.

In the future, there are many areas that will require intensive exploration. How do different cell types interact during development? What is the circuit in the diagrams and what are the nodes of interaction? Do the pathways that regulate cell proliferation and survival in the embryo also control regeneration and cancer formation in the adult? What are the cancer GRNs and what are the differences between normal embryonic development and cancer formation? Can we design drugs that target specific points in the network to correct disorders and reverse cancer formation? The zebrafish is a well-established model for addressing these questions.

Because of its many unique advantages, including transparent embryos and mutant adults, rapid embryonic development, short sex maturation time, large numbers of progeny, and well-developed gene transfer technology, zebrafish has become a popular research model for genetic and developmental biology. In the past 3 decades, several large-scale genetic screens and the nearly complete zebrafish whole-genome sequencing have considerably increased the use of zebrafish in diverse research areas. Thus, the zebrafish model has become an established and evolving system for liver cancer. Several important technologies have been established in zebrafish, including Tol2 and MultiSite gateway-based construction for gene analysis, as well as zebrafish xenotransplantation methods. Tumor xenografting has been recently developed in zebrafish to complement and overcome the deficiencies of other model systems. As an established animal disease model, the zebrafish pro-
vides a great opportunity to test the functions of disease markers in vivo and can be an effective and efficient system for drug screening. Research using zebrafish can integrate basic research with animal disease models and clinical research for successful implementation in biopharmaceutical industries.

REFERENCES


LIVER DEVELOPMENT AND CANCER FORMATION IN ZEBRAFISH


