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TOPIC HIGHLIGHT

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Zebrafish as a disease model for studying human hepatocellular carcinoma

Jeng-Wei Lu, Yi-Jung Ho, Yi-Ju Yang, Heng-An Liao, Shih-Ci Ciou, Liang-In Lin, Da-Liang Ou

Jeng-Wei Lu, Yi-Ju Yang, Heng-An Liao, Liang-In Lin, Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei 100, Taiwan

Yi-Jung Ho, Institute of Preventive Medicine, National Defense Medical Center, New Taipei 237, Taiwan

Yi-Jung Ho, Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan

Shih-Ci Ciou, Institute of Biotechnology, National Tsing Hua University, Hsinchu 300, Taiwan

Liang-In Lin, Department of Laboratory Medicine, National Taiwan University Hospital, Taipei 100, Taiwan

Da-Liang Ou, Graduate Institute of Oncology, College of Medicine, National Taiwan University, Taipei 100, Taiwan

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Correspondence to: Da-Liang Ou, PhD, Graduate Institute of Oncology, College of Medicine, National Taiwan University, 5F, No.2, Xuzhou Road, Taipei 100, Taiwan. dlou@ntu.edu.tw
Telephone: +886-2-23123456-88662
Fax: +886-2-33936523

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Abstract

Liver cancer is one of the world's most common cancers and the second leading cause of cancer deaths. Hepatocellular carcinoma (HCC), a primary hepatic cancer, accounts for 90%-95% of liver cancer cases. The pathogenesis of HCC consists of a stepwise process of liver damage that extends over decades, due to hepatitis, fatty liver, fibrosis, and cirrhosis before developing fully into HCC. Multiple risk factors are highly correlated with HCC, including infection with the hepatitis B or C viruses, alcohol abuse, aflatoxin exposure, and metabolic diseases. Over the last decade, genetic alterations, which include the regulation of multiple oncogenes or tumor suppressor genes and the activation of tumorigenesis-related pathways, have also been identified as important factors in HCC. Recently, zebrafish have become an important living vertebrate model organism, especially for translational medical research. In studies focusing on the biology of cancer, carcinogen induced tumors in zebrafish were found to have many similarities to human tumors. Several zebrafish models have therefore been developed to provide insight into the pathogenesis of liver cancer and the related drug discovery and toxicology, and to enable the evaluation of novel small-molecule inhibitors. This review will focus on illustrative

examples involving the application of zebrafish models to the study of human liver disease and HCC, through transgenesis, genome editing technology, xenografts, drug discovery, and drug-induced toxic liver injury.

Key words: Cancer model; Hepatocellular carcinoma; Liver disease; Zebrafish; Drug screening

Core tip: Hepatocellular carcinoma is one of the major cancers in the world and involves multiple mechanisms of tumor formation. Recently, the zebrafish has gained acceptance as a platform for developmental biology, drug toxicology, and translational medical research, offering innovative methods for studying disease and cancer formation. In this article, we summarize recent advances in the study of HCC based on the zebrafish as a model system through the use of transgenesis tools, genome editing technology, xenografts, drug hepatotoxicity, and novel drug discovery. Finally, we emphasize how each system works and how the technology was used in this cancer model.

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INTRODUCTION

Liver cancer is one of the world's most common cancers and the second leading cause of cancer deaths, with nearly 745000 deaths recorded in 2012[1]. The incidence of liver cancer is higher among men than women[2]. Hepatocytes are the main cells to constitute 70%-85% of the liver mass and are responsible for the metabolism of carbohydrates, amino acids, lipids, and chemical compounds as well as for the maintenance of the physiological environment[3-5]. Hepatocellular carcinoma (HCC), a primary hepatic cancer, accounts for 90%-95% of liver cancer cases. The pathogenesis of HCC consists of a stepwise process of liver damage that extends over decades, due to hepatitis, fatty liver, fibrosis, and cirrhosis before developing fully into HCC. Chronic liver damage induces genetic alterations of the hepatocytes, leading to cell death, cellular proliferation, dysplasia, and neoplasia. Multiple risk factors are highly correlated with HCC, including infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, aflatoxin exposure[2,6], and metabolic diseases[7] (Figure 1). These factors play critical roles in regulating multiple oncogenes or tumor suppressor genes and activating tumorigenesis-related pathways.

Persistent viral infections are the critical cause of HCC formation. Statistically, 57% of cirrhosis cases and 78% of HCC cases result from HBV and HCV infection[8]. HBV infection causes chromosome instability or insertional mutagenesis[9]. In particular, the HBV X protein (HBx), a small peptide with a molecular mass of approximately 17 kDa, is vital in the pathogenesis of HCC and becomes a prognostic marker of HBV infection and HCC. HBV infection plays an important role in the development of the tumor microenvironment in HCC by regulating the accumulation and activation of both cellular components, such as immune cells and fibroblasts, and non-cellular components of the microenvironment, such as cytokines and growth factors. HBV thus significantly affects the progress of the disease and prognosis[10]. HBx is able to enhance HBV replication, interfere with host gene transcription, interrupt protein degradation, regulate signaling pathways, and deregulate the cell cycle to manipulate cell death[11]. Numerous studies[12,13] have confirmed that the overexpression of HBx causes HCC.

HCV infection is the main risk factor for HCC in developed countries, accounting for approximately one-third to half of all cases. HCV infection leads to activation of Notch and Toll-like receptor pathways in cirrhosis, deregulation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway in early carcinogenesis, and upregulation of DNA replication/repair genes and the cell cycle in the late cancerous stages[14]. HCV proteins such as the core protein and nonstructural protein 5A interact with host cells to regulate processes such as cell signaling, transcriptional modulation, apoptosis, and endoplasmic reticulum stress[15]. A large number of HCV-infected persons develop chronic HCV infection, which can lead to liver fibrosis, cirrhosis, and HCC[16].

Alcohol is a co-carcinogen and is synergistic with the above risk factors for liver cancer. Cirrhosis is observed to have a high correlation with alcohol-associated HCC. Alcohol activates the JAK/STAT and p38 mitogen-activated protein kinase (MAPK) pathways, which responds by producing cytokines, chemokines, and stress. These changes, in turn, affect cell differentiation and growth[17]. Acetaldehyde, a metabolite of alcohol, is even considered to be a carcinogen as it increases oxidative stress and damages DNA[18]. These effects may induce liver fibrosis and cause cirrhosis and HCC development. However, approximately 5%-30% of HCC patients lack any apparent identifiable risk factors for their cancer. Non-alcoholic fatty liver disease (NAFLD) is the hepatic component of metabolic syndromes such as insulin resistance, obesity, hypertension, and hyperlipidemia; it includes both simple steatosis and non-alcoholic steatohepatitis (NASH)[19]. NAFLD/NASH itself becomes a risk factor for HCC, even in the absence of cirrhosis, because insulin resistance and inflammation are involved in HCC carcinogenesis.

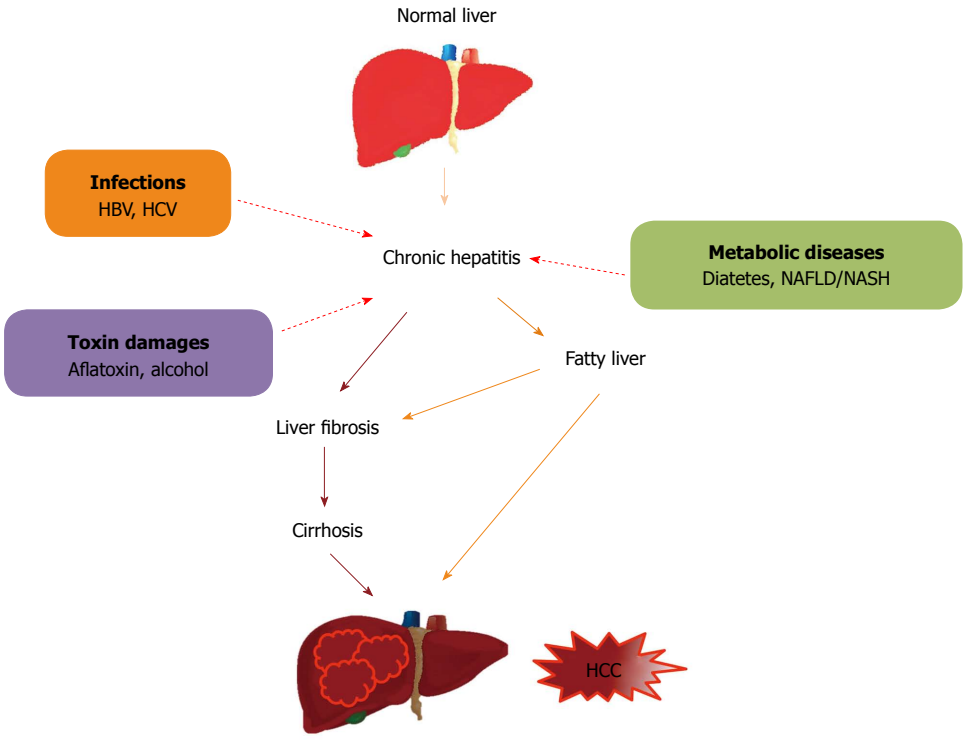

Figure 1 Pathogenesis and risk factors of hepatocellular carcinoma. Hepatocellular carcinoma (HCC) formation results from multiple risk factors, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol, Aflatoxin, and metabolic diseases. These risk factors induce chronic hepatitis, which activates inflammatory pathways. After decades, this inflammatory stress leads to DNA damage and cell cycle dysregulation in hepatocytes, which eventually leads to the development of HCC from chronic disease states, such as liver fibrosis and cirrhosis. Metabolic diseases progress into fatty liver diseases. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Aflatoxin, especially Aflatoxin B1/AFB1, is a genotoxic hepatocarcinogen and a kind of co-carcinogen. AFB1 is metabolized by cytochrome-P450 enzymes to become less harmful metabolites. However, aflatoxin B1-8,9-epoxide (AFBO), the reactive intermediate chemical compound, is a highly reactive genotoxic compound. AFB1 and AFBO both bind to liver cell DNA and form DNA adducts, causing DNA strand breakage, DNA base damage, and oxidative damage[6,20]. AFB1 has been found to accelerate the development of HCC initiated by other risk factors.

The zebrafish has become a model organism exploited in life science fields including embryonic development, toxicity, cancer research, human diseases, and drug screening[21,22]. The genome of the zebrafish is comprised of 25 chromosomes, which contain the full set of genes homologous to other vertebrates. After the current zebrafish genome was fully sequenced, approximately 70% of its orthologous genes were found to be associated with human disease[23,24]. Zebrafish have the following advantages and disadvantages: a short life cycle, low maintenance costs, small space requirements for maintenance, a large number of offspring, immune system deficiencies in early zebrafish embryos, transparency and transgenic lines, lower numbers of cells required for xenotransplantation per animal, availability for high-throughput drug screening, small organs and blood vessels, low body temperature, and a lack of organs such as lungs, among others. Compared to the mouse model, zebrafish are inexpensive and can be easily used to rapidly create a transgenic animal model. Transgenes can be controlled by ubiquitous, inducible, or tissue-specific promoters. Furthermore, transparent zebrafish carry fluorescent proteins that allow the visualization of specific cells in real time. Such capabilities enable investigators to observe and trace specific cells and to produce a spatiotemporal analysis of gene expression. Therefore, the zebrafish is a suitable option for monitoring transgenic tumors from initiation, progression, and metastasis to transplantation. In addition, the zebrafish model can be used for large-scale genetic and high-throughput screening[25,26]. These attributes make the zebrafish a more flexible option among animal models amenable to liver disease studies.

The genes and pathways involved in hepatogenesis and liver cancer are largely conserved between zebrafish and humans[27]. Hepatocytes possess similar functions in zebrafish and mammals and demonstrate similar genesis for shared histopathological characteristics such as steatosis, cholestasis, and neoplasia[28]. Expression of HCV core protein in transgenic zebrafish treated with thioacetamide (TAA) was the first application of zebrafish in HCC studies.

Pathological features observed in this transgenic zebrafish model include steatohepatitis, fibrosis, cirrhosis and HCC. Progression to HCC is reduced to six weeks relative to TAA-treated wild type zebrafish and

becomes a powerful preclinical platform for studying the mechanism of hepatocarcinogenesis in evaluating therapeutic strategies for HCC[29]. Today, genome editing technologies are rapidly advancing. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems have been developed to rapidly induce targeted genetic modifications[30]. Such technologies promote the generation of transgenic animal models. This review focuses on the zebrafish model for HCC. We summarize liver development and the anatomy of the zebrafish. Subsequently, expression systems and genome editing technologies are presented to examine the current status of transgenic zebrafish development. Finally, the zebrafish models for liver disease and HCC are introduced for a better understanding of recent findings regarding mechanisms, drug screening, and drug-induced toxic liver injury.

OVERVIEW OF ZEBRAFISH LIVER DEVELOPMENT AND ANATOMY

The liver is a critical organ for vertebrates. Hepatocytes, the cell type which constitutes the majority of the liver, play a major role diverse biological functions, including digestion and metabolism through the regulation of many essential nutrients, the storage of vitamins, the decomposition of red blood cells, the synthesis of plasma proteins such as prothrombin, fibrinogen, and albumins, the production of hormones, and detoxification in mammals[31,32].

The zebrafish is effective as an animal model for studying liver development because of its experimental advantages, as has been demonstrated in numerous publications. However, some characteristics of the zebrafish liver differ from other vertebrates and mammals. For example, hepatocytes are not noticeably organized in cords or lobules, and the typical portal triads are not obvious in the zebrafish liver. The portal veins, hepatic arteries, and large biliary ducts of zebrafish are spread randomly within the hepatic parenchyma and are not grouped into portal tracts as in the mammalian system. Moreover, the hepatocytes are arranged as tubules that surround small bile ducts rather than as bilayered hepatocyte plates as in the mammalian system. The intrahepatic bile ducts are derived from the bile canaliculi. The bile ducts fuse and ultimately form the gallbladder. The bile is collected in the gallbladder *via* large ducts and an extrahepatic biliary system. Moreover, there are no Kupffer cells in the zebrafish liver[33,34]. The liver of zebrafish contains three lobes, one ventral and two lateral, which lie along the intestinal tract. The liver of teleosts is similar to the mammalian liver and plays a central role in metabolic homeostasis, including the processing of carbohydrates, proteins, lipids, and vitamins. In addition, it also plays an important role in detoxification and the synthesis of serum proteins, including albumin, fibrinogen, complement factors, and acute-phase proteins[34].

Similar to the process in the mammalian liver, hepatogenesis occurs in three major phases in zebrafish: hepatoblast specification, budding/differentiation, and hepatic outgrowth, accompanied by morphogenesis[28,35,36]. Cells in the anterior endodermal rod develop into hepatoblasts at 22 h post-fertilization (hpf) through the expression of *hhex* and *prox1* during the hepatoblast specification phase. Hepatoblasts are located on the left side of the anterior gut tube, and the liver bud begins to form between 26 and 28 hpf. Some marker genes, such as ceruloplasmin (cp) and transferrin, are expressed in the liver bud at 32 hpf, with the clear emergence of the liver primordium at 48 hpf during the differentiation phase. The liver bud leaves the intestine at approximately 50 hpf. Due to proliferative acceleration, hepatic outgrowth begins between 60 and 72 hpf and continues until the liver attains its apposite size, and a rapid growth phase of the liver begins at 80-84 hpf[37]. During the end of the outgrowth phase (120 hpf), the liver relocates from the left side to the right side[28].

Signaling molecules and transcription factors are conserved in hepatogenesis between mammals and zebrafish. These pathways also regulate early liver development in zebrafish, though there are some differences. FGF, BMP, and WNT signaling pathways are crucial for hepatogenesis[38,39]. FGF signaling is critical for hepatic specification in zebrafish and mice. Overexpression of a dominant negative FGF-receptor in zebrafish embryos between 18 and 26 hpf decreases the later expression of *hhex*, *prox1*, *gata4*, *gata6,* and cp[40]. A previous study revealed that BMPs are vital for zebrafish hepatic specification: zebrafish mutations such as lost-a-fin or over-expression of a dominant negative BMP receptor led to reduced expression of some hepatic specification genes, such as *hhex* and *prox1*[40]. Induction of *wnt* expression led to a block in liver specification in early somitogenesis and created an enlarged liver after a few hours in zebrafish liver development[41].

Hepatogenesis is a complex process controlled by many transcription factors. The earliest conserved liver-specific transcription factors regulating hepatogenesis, such as *hhex* and *prox1,* are initially expressed and play crucial roles in the zebrafish hepatic bud at 24 hpf[40,42-44]. These hepatic nuclear factors also participate in liver development and differentiation in mammalian hepatogenesis[45]. Some transcription factors, including *sox17*, *foxa1*, *foxa2*, *foxa3*, and *gata* family members are required for both the generation of endoderm and the liver bud[35,46,47]. Recent research indicates that several genes, such as liver-enriched gene 1 (*leg1*), play important roles in the outgrowth stage in zebrafish[48]. Such research has also revealed that hypoxia-inducible transcription factors, such as hif2-alpha, directly regulate the hepatic outgrowth

phase through binding to the promoter region of *leg1* but do not directly regulate the liver specification phase in zebrafish embryos[49].

According to the latest data, there is evidence that epigenetic regulation of gene expression in zebrafish liver development also plays an important role. Histone acetylation (*hdac*) and DNA methylation (*dnmtin*) are two major mechanisms regulating gene expression. An analysis of mutations in *hdac* or *dnmtin* embryos demonstrated that epigenetic regulation controls both hepatic specification and outgrowth phases in zebrafish liver development. For example, *hdac1* mutants develop small livers as a result of hepatic patterning defects[50]. Embryos treated with an *hdac* inhibitor at 24 hpf have also been shown to develop a small liver due to inhibition of *hhex* and *prox1* gene expression. Furthermore, the knockdown of *hdac1* and *hdac3* results in multiple defects in embryos. Aberrant *hdac3* is more specific for liver development as it regulates zebrafish liver growth by suppressing growth differentiation factor 11, a member of the TGF-β family of growth factors[51]. Ubiquitin-like with PHD and RING finger domains 1 (*uhrf1*) plays a role in DNA methylation by recruiting DNA methyltransferase 1 (*dnmt1*) to hemimethylated DNA. The *uhrf1* mutants have defects in zebrafish hepatic outgrowth[52].

CONSTITUTIVE AND INDUCIBLE EXPRESSION SYSTEMS FOR THE DEVELOPMENT OF HCC MODELS IN ZEBRAFISH

Over the past 25 years, the available zebrafish transgenic technology has advanced significantly[53,54]. Transgenesis is an essential technique as in other model organisms. A variety of transgenic expression systems exist for zebrafish, including constitutive and inducible systems. Initial transgenes were plasmid-based with ubiquitous promoters driving the expression of reporter genes and demonstrated that transgenic technology was a viable, reproducible strategy in zebrafish. In recent years, the use of transgenesis in zebrafish has become widespread. Researchers have used a mammalian promoter, promoters from other fish species, and tissue-specific promoters to drive gene expression[55]. In general, the design of most transgenic vectors thus far incorporates a single promoter to control when and where a transgene is expressed. The frequency of the development of germline founders is associated with the method of the introduction of the DNA. Supercoiled[53,56] or linear DNA[54] injection yields 1%-10% germline transgenic founders, while linearized ISce-I[57] meganuclease yield 20%-30%. The rate of transgenesis has recently seen a dramatic increase with the use of transposon-based systems: 30% with Sleeping Beauty[58] and Ac/Ds[59], and 50% with Tol2[60,61]. The Tol2 element is an active transposable element found in *Medaka* genomes, and subsequent production of some cloning vectors has facilitated the use of this element in zebrafish, allowing the generation of many transgenic fish lines[62]. The Tol2 element transposon can be efficiently excised and integrated into the zebrafish genome using coinjection with *Tol2* mRNA and vector plasmid[60]. Cloning vectors from multiple sources, including mini inverted repeat transposons and Tol2 transposase transcription vectors, made use of multisite Gateway cloning vectors[63,64]. In particular, the Tol2-kit was established for the scientific community to allow the use of versatile vectors. Gateway cloning technology is a universal cloning method based on the att site-specific recombination properties of bacteriophage lambda and enables the rapid and highly efficient transfer of DNA sequences into multiple vector systems for protein expression and functional analysis[65]. The online Tol2-kit community (http://tol2kit.genetics.utah.edu/index.php/Main_Page) provides detailed information and has helped to make the Tol2 transposon system a routine genetic engineering tool. Widely useful entry clones were created by combining heat-shock protein 70 (hsp70), CMV/SP6, histone2A-X, β-actin, and upstream activating sequence (UAS) promoters, cytoplasmic, nuclear, membrane-localized fluorescent proteins and Gal4VP16, IRES-driven GFP cassettes, and two Tol2-based destination vectors, one with a Cmlc2/GFP transgenesis marker[64]. One of the most useful GFP transgenic fish lines was derived with a zebrafish liver fatty acid-binding protein (L-FABP) promoter[66,67]. A zebrafish model for hepatocarcinogenesis has been since developed through the expression of oncogenes under the control of the L-FABP promoter. Liver-specific expression of HBx, *src*, and endothelin 1 (*edn1*) established with Tol2 methodology triggered hepatocarcinogenesis in zebrafish[68,69].

While previous studies demonstrated the utility of constitutive expression systems, constitutive expression of oncogenes is often found to lead to gross tumor development which can result in embryonic lethality. Inducible systems can avoid these potentials deficiencies in constitutive systems as the duration and dosage of gene expression can be monitored, thus allowing for the spatiotemporal control of oncogene expression. Inducible systems currently being used include Heat-shock, Cre-loxP, GAL4-UAS, Tet-On, Tet-Off, and Mifepristone systems[70] (Table 1). Heat-shock proteins were originally identified in cells after exposure to environmental stress. Induced jumps in temperature have been used to achieve spatiotemporal control of transgene expression in zebrafish embryos. GFP linked to an hsp70 promoter has been used to establish the pattern of gene expression induced by heat shock. At a normal temperature, GFP expression in transgenic embryos was not detectable. However, single embryos heat-shocked by exposure to 38 ℃ for 30 min exhibited GFP expression in approximately 20%-90% of cells for more than 24 h after heat

Table 1 Advantages and disadvantages of constitutive and inducible expression systems

Expression systems	Advantages	Disadvantages
Constitutive	Well established, commercially available; *in vitro* and *in vivo*, successful methodology for expression of transgene	Expression of oncogenes may cause advanced/highly aggressive tumors and early lethality
Heat-shock	Expression of transgene can be induced on a single cell level	Adverse effects that may arise from the heat shock
Cre-loxP	Well established; commercially available; *in vivo*, successful methodology for expression of transgene	Not all tissue specific promoters are perfectly specific; leaky gene expression; two plasmid system
GAL4/UAS	Well established; *in vivo*, successful methodology for expression of transgene	*In vivo* expression of GAL4 can have side effects, probably related to immune and stress responses; two plasmid system
Mifepristone	Well established; *in vivo*, successful methodology for expression of transgene	Opening and closing of the switch is slow (hours to days); cell permeability of the RU-486 can be restricted
Tet-on/off-inducible	Well established; commercially available; *in vitro* and *in vivo*, successful methodology for expression of transgene	Opening and closing of the switch is slow (hours to days); cell permeability of the doxycycline can be restricted; two plasmid system

treatment in a variety of tissues types[71,72].

Multiple transgenic lines have been derived in zebrafish through the use of tissue-specific expression of Cre recombinase. Initially, a plasmid-based system was developed for detecting Cre expression *in vivo*[73]. A neural progenitor-specific (nestin) promoter was used to drive the expression of an mCherry gene, flanked by loxP sites, and upstream of a promoterless EGFP-fused to zebrafish *kras-V12* oncogene, resulting in the exclusive expression of mCherry. Once this plasmid was exposed to Cre recombinase, the mCherry gene was excised, and the EGFP gene, fused to the oncogene, was controlled by the nestin promoter[74]. The GAL4-UAS system has also been successfully exploited in zebrafish to misexpress genes in a tissue-specific manner. The GAL4-UAS methodology requires two transgenic lines: the activator zebrafish line which expresses the yeast transcriptional activator GAL4 under the control of a specific promoter, and the effector zebrafish line which possesses the transgene of interest fused to the DNA-binding motif (UAS) of GAL4[75,76]. In 1999, an activator line was developed to express GAL4 under the control of the β-actin promoter. In these experiments, the transgene in the effector line encoded a *myc*-tagged protein adjacent to the UAS of GAL4[77]. This report demonstrated that the cross of the effector line with an activator line is necessary for gene expression. This strategy was used to develop an HCC model. In this model, walleye dermal sarcoma virus *rv-cyclin* gene (*orf-A*) fused to the UAS of GAL4 was expressed in the livers of zebrafish when crossed to animals harboring GAL4 under the control of L-FABP promoter[78].

Chemically inducible expression systems (Tet-On, Tet-Off, and Mifepristone) have also been used in zebrafish[79-82]. The Tet-On and Tet-Off systems are binary transgenic systems in which the expression of a transgene is dependent on the activity of an exogenous inducible transcriptional activator. In both the Tet-On and Tet-Off systems, expression of the transcriptional activator can be regulated both reversibly and quantitatively by exposing the transgenic animals to varying concentrations of tetracycline derivatives, such as doxycycline (Dox). The design of the Tet-On and Tet-Off systems allows tissue-specific promoters to drive the expression of the reverse Tet-controlled transcriptional activator (rtTA) and Tet-controlled transcriptional activator (tTA), resulting in tissue-specific expression of the regulated target transgene[55].

Several HCC models have been developed using such technology. Li *et al*[80,83] fused the xiphophorus *xmark* and mouse *myc* oncogenes to the rtTA responsive element, and placed the rtTA transgene was under the control of the 2.0-kb L-FABP promoter. Liu *et al*[81] fused the HBV and HCV oncogenes to the tTA responsive element, and placed the tTA transgene under the control of the 2.8-kb L-FABP promoter. In the mifepristone inducible LexPR system, the LexPR chimeric transactivator was fused to a 2.0-kb L-FABP promoter to produce the driving zebrafish line, and the effector zebrafish line contained EGFP-fused to zebrafish *kras-V12* oncogene under the control of the LexA-binding site. Expression was induced by exposing animals to varying concentrations of mifepristone (RU-486)[81]. In these studies, dose-dependent, Dox, or mifepristone mediated activation of oncogene expression were detected in the liver of the transgenic zebrafish.

GENOME EDITING TECHNOLOGY FOR GENE KNOCKOUT AND LOSS OF FUNCTION IN ZEBRAFISH

Over the past two decades, genetic engineers have made great strides in developing a reliable technique to examine genotypes. Gene knockdown using small interfering RNAs and microRNAs restore the function of dysfunctional genes, but the main disadvantages are off-target interactions and the temporary nature of inactivation achieved through these methods. Today, ZFNs, TALENs, and CRISPR/Cas have become well-established genome editing tools for customizing genomes in human, animal, and plant cells[83-85]. The characteristics and gene editing capabilities in

Table 2 Characteristics of three genome editing systems

Nucleases	ZFN	TALEN	CRISPR/Cas
DNA binding domain	Multiple zinc finger peptides	Transcription-activator like effectors	CRISPR-derived RNA/Single-guide RNA
Endonuclease	Fok1	Fok1	Cas9
Binding specificity of each repeat	3 bp	2 bp	1 bp
Target site length	18 to 36 bp	30 to 40 bp	23 bp
Off-target	High probability	Low probability	Variable
Libraries generation	No	Feasible, depend on technology	Yes, cloning 20 bp, oligos targeting each gene into a plasmid

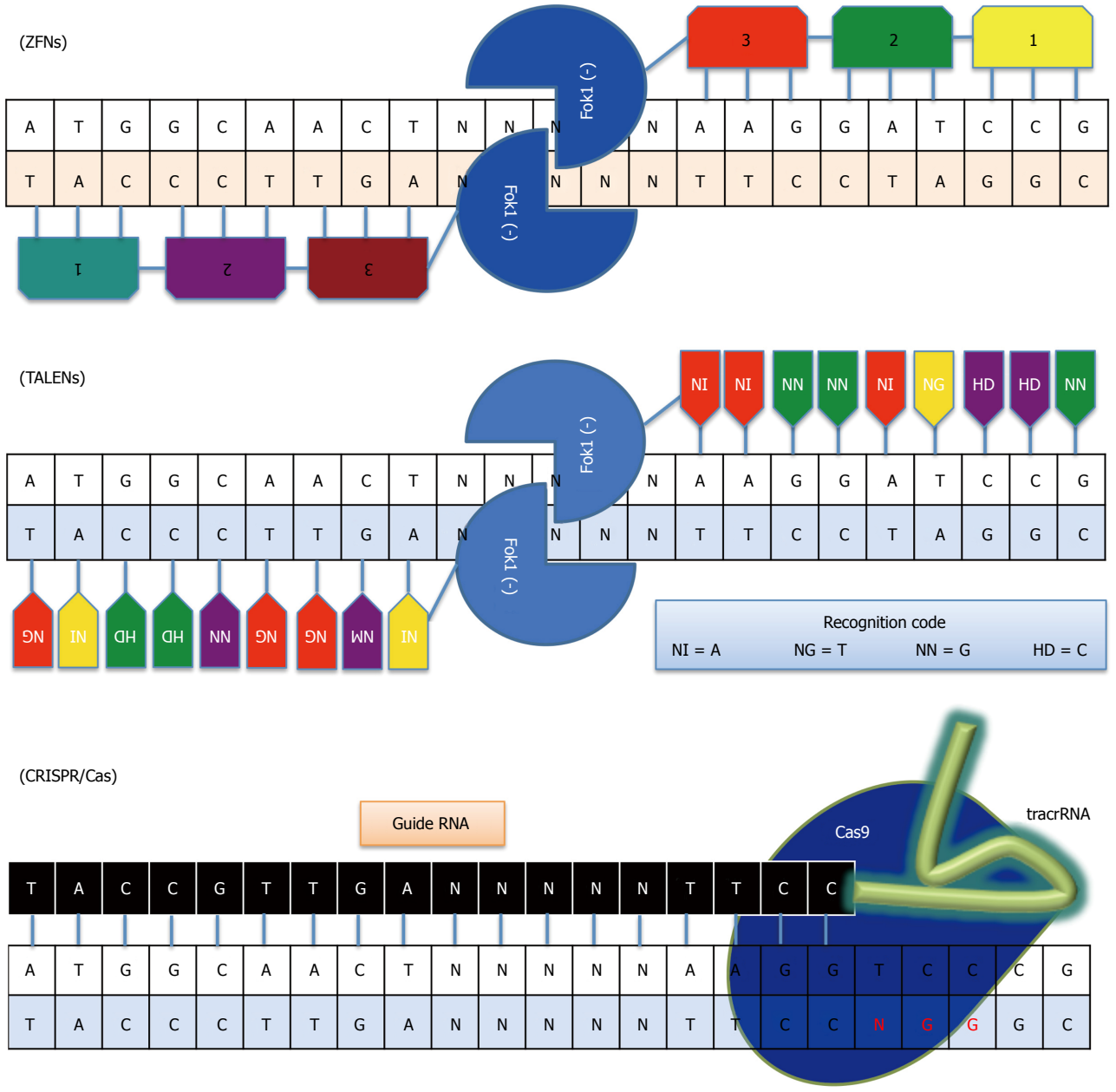

Figure 2 Schematic representation of programmable engineered nucleases of ZFNs, TALENs and CRISPR/Cas.

complex genomes of ZFNs, TALENs, and CRISPR/Cas systems are summarized in Table 2 and Figure 2. RNA-programmable DNA nucleases have been adapted as a precise genetic scissors for correcting and editing genetic defects[86]. Site-specific nucleases induce DNA double strand breaks (DSBs) that stimulate non-homology end joining (NHEJ) and homology-directed repair (HDR) for targeted genomic loci[2].

As one of the numerous DNA-binding motifs in eukaryotic genomes with the ability to recognize

any sequence[87,88], ZFNs are being widely applied to anything in biological research, from the design of animal models to human gene therapies[89]. A ZFN is composed of two domains: a site-specific DNA-binding domain, which is derived from a zinc finger containing transcription factor, and a bacterial Fok1 restriction enzyme endonuclease domain. The zinc finger protein recognizes a 3-bp sequence of DNA on the major groove, with its tandem repeats potentially attaching to a stretch of nucleotides between 9 and 18 bp long[90].

To perform site-specific cleavage of DNA, two ZFN monomers are necessary for the process; one monomer recognizes the binding site on the forward strand while the other recognizes it on the reverse strand. The ZFN binding on both strands enables higher specificity targeting and dimerization of Fok1 in an adequate space[91], so that the pair of Fok1 nuclease domains can cleave the DNA generating a DSB. Cells then utilize either NHEJ or HDR to repair DSBs. The manner in which NHEJ introduces frameshifts into the coding region to knock out a gene, achieved for example through nonsense-mediated mRNA transcript disintegration, is not especially efficient. HDR, however, is used to generate a specific mutation by means of a repair template containing the desired mutation-paired oligonucleotide[92].

Zinc finger proteins with diverse binding specificities are designed using several methods. Modular assembly, which involves a preselected library comprising zinc finger domains for the recognition of 64 nucleotide triplets, is one way of generating customized zinc finger domains[93]. To identify the perfect combination, oligomerized pool engineering utilizes a zinc finger array, and through bacterial-based selection, identifies proteins that bind efficiently to the target site[94]. Other strategies also apply zinc finger modular assembly based on context-dependent DNA to produce ZFNs with endonuclease (endogenous) activities[95]. Although ZNFs offer convenience and are widely utilized, they still possess a high off-target effect[96], which may be improved by developing a heterodimer composed of ZFNs with different Fok1 domains to cleave target DNA[97-99].

TALENs contain a DNA-binding domain and a Fok1 catalytic domain, just like ZFNs, for genomic engineering. A DNA-binding domain is constructed with an N-terminal segment, a central repeat domain, and a half repeat. The central repeat domain is comprised of several monomers that are called transcription activator-like effectors (TALEs). TALEs are effector proteins that are secreted from the bacteria of the *Xanthomonas* genus. They were first found in plant cells, enhancing their susceptibility to pathogens[100]. TALEs are tandem repeats of a 34-amino-acid domain[101], and positions 12 and 13 are known as repeat-variable di-residue (RVD) domains used to determine the specificity of the TALEs. There are four RVD domains, NN, NI, HD, and NG, for the recognition of guanine, adenine, cytosine, and thymidine, respectively[102]. TALEs function as eukaryotic transcription factors *via* DNA binding to activate target gene expression. Fok1 is located in the C-terminal segment and generates a DSB in a spacer sequence. TALENs therefore can be used for targeted gene disruption[103]. It is challenging to construct TALE repeats because each TALE repeat unit has high similarity. Specific methods, such as the restriction enzyme and ligation method[104], Golden Gate cloning[105], and fast ligation-based automated solid-phase high-throughput system[106] have all been designed for the rapid assembly of specific TALENs, so that custom-designed TALENs are in fact a realistic possibility for genetic engineering.

CRISPR/Cas is a prokaryotic defense system against invasion of foreign DNA, utilizing an RNA-guided DNA cleavage system. Small fragments (protospacers) of foreign DNA are inserted at repeat sequences in their own genomes to form CRISPR[107]. The type Ⅱ system consists of a trans-activating crRNA (tracrRNA) in addition to the primary CRISPR RNA transcript (pre-crRNA) transcribed from the protospacers, which is subsequently processed into short crRNAs[108]. To achieve direct sequence-specific DNA recognition and cleavage, CRISPR-associated protein 9 (Cas9) must be complexed with both the crRNA and the tracrRNA, with the crRNA providing the sequence required for target recognition through Cas9. The essential targeting component (5'-NGG-3' protospacer adjacent motif sequence) is located upstream of the crRNA, which is recognized through the Cas9. Through this mechanism, CRISPR/Cas systems cleave the target DNA sequence of 23 bp. Compared to ZFNs and TALENs, the generation of a CRISPR/Cas target specific endonuclease is much easier with the methods of cloning and transcription[109]. Recently, a CRISPR/Cas9 construct was established for tissue-specific gene disruption in zebrafish, and this vector system may become a unique tool to spatially control targeted somatic mutations, gene knockout and loss of function studies in zebrafish[110].

HCC AND LIVER DISEASE MODELS IN TRANSGENIC ZEBRAFISH

The most studied oncogene associated with the development of HCC is the HBx antigen from HBV. HBx has been shown to induce HCC in mice and enhance colony formation in HCC cell lines[111-113]. Transgenic mouse models indicate that HCV is directly pathogenic and oncogenic[114,115]. AFB1 is one of the most prominent carcinogens associated with HCC[116], and it is known to induce formation of DNA adducts and *p53* mutations in liver cell lines[117]. Mutational inactivation of *p53* has been described as one of the key molecular mechanisms involved in the pathogenesis of HCC[118]. AFB1 is synergistic with other factors as AFB1 treatment

induced significantly more liver tumors in HBx and HCV transgenic mice than in wild-type mice[114,115].

Models for liver disease and HCC have been generated in zebrafish through the tissue specific expression of such oncogenes regulated by the L-FABP promoter. In zebrafish, HBx overexpression causes hepatic fat accumulation and liver degeneration in a wild-type background[119]. Tumorigenesis however requires inactivation of the *p53* tumor suppressor pathway, either through mutation of the gene itself or aberrant expression of a negative regulator, such as murine double minute 2 (*mdm2*) protein. Overexpression of *mdm2* alone in the zebrafish liver leads to growth retardation and a fragile liver[120]. Another oncogene affecting the p53 pathways is *gankyrin.* This protein binds ubiquitin protein ligase *mdm2* which promotes *p53* degradation. The inhibition of p53 function through any of these mechanisms prevents the activation of *p53*-dependent apoptotic genes, which leads to cell survival, genomic instability, and oncogenic transformation[121]. Overexpression of *gankyrin* was found to induce hepatic steatosis and regulated miR-16, miR-27b, miR-122, and miR-126. The protein has also been shown to be involved in lipid metabolism[122].

UHRF1 is an important regulator of DNA methylation that is highly expressed in many cancers. *UHRF1* overexpression destabilizes and delocalizes *dnmt1,* causing DNA hypomethylation, *p53*-mediated senescence, and hepatocarcinogenesis in zebrafish[123]. Cyclins are involved in tumor formation and cell death. *rv-cyclin* may also play a role in walleye dermal sarcoma tumor regression by inducing apoptosis[124,125]. Liver-specific expression of walleye dermal sarcoma virus *rv-cyclin* (*orf-A*) in zebrafish protects the fish liver from damage with treatment of 7,12-Dimethylbenz[a]anthracene and delays the onset of malignancy[78].

Edn1 has been identified as a gene that is significantly up-regulated in HBx-induced HCC in the mouse model[126]. Liver-specific induced expression of *edn1* caused steatosis, bile duct dilation, hyperplasia, and HCC in zebrafish[68]. Expression of the transcription factor Yin Yang 1 (*YY1*) was also significantly up-regulated by HBV in a concentration-dependent manner[127]. A previous study has demonstrated through chromatin immunoprecipitation that HBx interacts with YY1[128]. CCAAT/enhancer-binding protein alpha which controls differentiation of hepatocytes was found to be a direct target down-regulated by YY1[129]. Overexpression of *YY1* promoted zebrafish liver steatosis and lipotoxicity by inhibiting C/EBP homologous protein 10 expression[130].

Excessive food intake and increased weight gain to the point of obesity is one of the causes of steatosis. Activation of cannabinoid receptor 1 (CB1R) is a molecular mechanism underlying the regulation of food intake, weight gain, and obesity in mammals. Tet-Off conditional expression of the zebrafish *CB1R* ortholog gene promoted hepatic lipid accumulation and lipotoxicity through the induction of *srebp-1c* expression in zebrafish[131]. In vertebrates, apoptosis is a fundamental part of normal embryonic development and participates in sculpting organs and regulating cell populations. *zfBLP1* and *zfMcl-1a* are functionally similar to members of the *Bcl-2* family, which inhibit apoptosis. Overexpression of *zfBLP1* or *zfMcl-1a* in zebrafish larval liver induced hyperplasia[132].

Combined treatment of zebrafish with HBx and AFB1 induced hepatitis, steatosis, and liver hyperplasia during the early stages of hepatocarcinogenesis[133]. HBx and *src* overexpression induced HCC in *p53* mutant zebrafish and revealed a role for *src* in HCC progression[69]. TAA enhanced the development of steatohepatitis, cirrhosis, and HCC induced by the expression of the HCV core protein in transgenic zebrafish[29]. *In vitro*, the HCV core protein has been shown to directly activate the RAS-RAF-MEK-ERK pathway[134]. In human HCC, Ras proto-oncogenes are activated in as many as 50% of all HCC cases, which leads to activation of downstream signaling pathways including RAF-MEK-ERK and PI3K-AKT-mTOR. Approximately 7% of HCCs carry activating mutations in the *K-RAS* oncogene, which is higher than the percentage of cases carrying *H-RAS* and *N-RAS* mutations. A high level of *kras-V12* expression induced through constitutive or inducible mechanisms initiated liver tumorigenesis in zebrafish[81,135]. The co-expression of HBx and the HCV core protein trigger intrahepatic cholangiocarcinoma in transgenic zebrafish[80]. However, transgenic zebrafish over-expressing HBx or HCV individually do not develop HCC.

Recently, expression of *kras-GV12* and *xmrk*, the homolog of mammalian epidermal growth factor receptor oncogene, in zebrafish with Tet-On conditional methodology has been reported as an outstanding model for revealing new therapeutic targets involved in oncogene-regulated hepatocarcinogenesis[79,136]. RNA sequencing analysis of an *xmrk* transgenic HCC model revealed a potential role for immune responses in HCC progression and regression. This model may provide molecular insight into the targeted inhibition and significance of immune response in tumor regression[137].

The liver is one of the most important organs for the study of autophagy[138]. In fact, liver tumors are one of the main phenotypes in knockout mice of autophagy-related genes[139]. In zebrafish, the EGFP-Lc3 transgenic line crossed with the *xmrk* transgenic line yielded animals susceptible to HCC and thus, demonstrated that autophagy plays an important role in HCC development[140]. Cross-species analyses demonstrated that Tet-On conditional expression of *myc* in a zebrafish model paralleled findings in *myc* mouse models for HCC. Elevated *myc* expression in zebrafish caused liver hyperplasia, adenoma, and HCC. *Myc*-induced liver tumors in zebrafish also possessed molecular signatures that were similar to those from

mouse and human HCC. This zebrafish model thus revealed a conserved role for *myc* in promoting hepatocarcinogenesis in all vertebrate species[82]. RNA expression profiling of liver tumors from the three different zebrafish models, *xmrk*, *kras-G12V,* and *myc,* showed however relatively little overlap in significantly deregulated genes and biological pathways. However, these three transgenic tumor signatures were found to be significantly correlated with advanced or late stage human HCC[141].

In human HCC, deregulation of MYC is frequently detected and correlated with poor prognosis. Two differentially expressed *MYC* orthologs exist in the zebrafish genome: *myca* and *mycb*. Overexpression of *myca* and *mycb* in the liver using a mifepristone-inducible system demonstrated that both *myc* genes were oncogenic. *myca* overexpression accelerated tumor progression and reduced apoptosis in *p53* mutant zebrafish. Malignant hepatocytes were dependent on sustained *myca* expression; withdrawal of the mifepristone inducer resulted in a rapid regression of HCC, with liver tumor regression occurring even in a *p53* mutant background[142].

RhoA is a member of the RHO small GTPase family, which is highly homologous to the RAS. These proteins are also involved in the regulation of cell cycle dynamics, and are key molecules for cell growth and tissue development of the switch. Expression levels and the overall activity of RhoA has been found to be elevated in HCC[143]. Tet-On conditional expression of *kras-G12V*, *rhoA*, constitutively active *rhoA-G14V,* dominant-negative *rhoA-T19N,* or *kras-G12V* plus one of the three *rhoA* genes, was also examined in zebrafish. Overexpression of *kras-G12V* during early development led to liver enlargement and hepatocyte proliferation. The increase in liver size was augmented by the dominant-negative *rhoA-T19N,* but abrogated by the constitutively active *rhoA-G14V*. This study revealed the existence of signaling crosstalk between *kras-V12* and *rhoA* in regulating liver overgrowth and hepatocarcinogenesis[136]. Based on these results, the zebrafish emerges as a model system for elucidating the mechanisms of hepatocarcinogenesis and for screening drugs to inhibit the oncogenic effects of specific genes (Table 3).

POTENTIAL APPLICATIONS OF XENOGRAFTS AND ZEBRAFISH HCC MODELS IN DRUG DISCOVERY

The United States Food and Drug Administration approves only a few new chemical entities for clinical usage each year because the investigation of new drugs is a lengthy and costly process. Drug-discovery generally proceeds first through *in vitro* assays, where cell proliferation, cytotoxicity, marker expression, motility, activation of specific signaling pathways, and changes in morphology are examined in response to treatment with small molecules[144], and second through *in vivo* screening where endpoints such as extended life span can be evaluated. The zebrafish has the advantage of combining both processes in a single model. It is a high-throughput and *in vivo* model simultaneously; therefore, the zebrafish might improve the success rate in the later stages of preclinical drug development while reducing the cost and the time necessary for the screening process[70].

The trend of using zebrafish embryos in screening for anti-cancer drugs continues to rise. The use of computational drug design and screening of zebrafish embryos has successfully uncovered a novel lead compound that displays selective inhibitory effects on CDK2 activity, cancer cell proliferation, and tumor progression *in vivo*[145].

Zebrafish/tumor xenograft models have been used to study angiogenesis, invasion, and metastasis. One advantage of zebrafish is that the embryos are transparent, allowing the observation of labeled tumor cells and the evaluation of response to candidate molecules in a high-throughput format *in vivo*[146]. In order to achieve maximum transparency, zebrafish embryos are incubated in an egg medium with 0.3% phenylthiourea to prevent the formation of pigments. (In the mouse system, the spatial resolution is limited *in vivo* due to normal opacification of the skin and subdermal structures). Tumor cells labeled with CM-DiI, a lipophilic fluorescent tracking dye, are injected into the perivitelline space or yolk of embryos at 48 hpf and are followed thereafter. *fli1*:gfp transgenic embryos and the whole-mount akaline phosphatase vessel staining assay allows for rapid and relatively easy investigation of tumor angiogenesis, cell dissemination, invasion, metastasis, and anti-vascular endothelial growth factor (VEGF) drugs for cancer therapy[68,147]. Transgenic zebrafish (*vegfr2*:grcfp) where GFP expression is restricted to blood vessels have been used to screen a compound library for antiangiogenic compounds. SU4312 and AG1478, two known anti-angiogenic compounds, were used as positive controls in the screen. Two new compounds with no previously described antiangiogenic activity, indirubin-3'-monoxime (IRO) and EM011 (9-bromonoscapine), were also identified[148,149]. Embryos of the transgenic *flk*:gfp zebrafish were also used in screening the compound library. One lead compound, rosuvastatin, was identified which could inhibit the growth of the zebrafish intersegmental vessels[150]. The zebrafish tumor xenograft model represents a new tool for investigating the neovascularization process and is exploitable for drug discovery as well as gene targeting in tumor angiogenesis.

In zebrafish HCC models, mifepristone-induced *kras-V12* transgenic larvae treated with MEK1/2 inhibitor PD98059 resulted in the inhibition of hyperplastic liver growth in 49% of cases. Inhibition of PI3K-AKT-mTOR signaling by LY294002 or rapamycin restored the normal liver phenotype in 57% and

Table 3 Zebrafish animal models of liver disease and hepatocellular carcinoma

Transgene name	Expression system	Liver pathology	Ref.
cnr1 (Zebrafish)	Tet-off-inducible	Steatosis	[132]
edn1 (Zebrafish)	Constitutive	Steatosis, bile duct dilation, hyperplasia and HCC	[69]
gankyrin (Zebrafish)	Constitutive	Atrophy, hypoplasia and steatosis	[123]
HBx (Human)	Constitutive	Hypoplasia and steatosis	[120]
HBx + *AFB1* (Human)	Constitutive	Hepatitis, steatosis and hyperplasia	[134]
HBx + *HCV* (Human)	Tet-off-inducible	Intrahepatic cholangiocarcinoma	[81]
HBx + $p53^{M214}$ (Human)	Constitutive	Chronic inflammation, steatosis, bile duct dilation, dysplasia and HCC	[70]
HBx + *src* (Human/Zebrafish)	Constitutive	Chronic inflammation, steatosis, bile duct dilation, dysplasia and HCC	[70]
HCV (Human)	Constitutive	Steatosis	[29]
HCV + *TAA* (Human)	Constitutive	Steatosis and HCC	[29]
kras-G12V (Zebrafish)	Mifepristone	Hyperplasia and HCC	[82]
kras-G12V (Zebrafish)	Constitutive	Hyperplasia and hepatocellular adenoma	[82]
kras-G12V (Zebrafish)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[137]
kras-G12V + $p53^{M214}$ (Zebrafish)	Constitutive	Hyperplasia and hepatocellular adenoma	[82]
kras-G12V + *RhoA* (Zebrafish)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[137]
kras-G12V + *RhoAG14V* (Zebrafish)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[137]
kras-G12V + *RhoAT19N* (Zebrafish)	Tet-on-inducible	HCC	[137]
Lc3 (Rat)	Constitutive	Investigation of liver autophagy	[141]
mdm2 (Zebrafish)	Constitutive	Atrophy, contraction and hypoplasia	[121]
MYC (Mouse)	Tet-on-inducible	Hyperplasia and hepatocellular adenoma	[83]
myca (Zebrafish)	Mifepristone	Small, typical, hypervascular and ascites of liver tumor	[143]
myca + *p53M214* (Zebrafish)	Mifepristone	Small, typical, hypervascular and ascites of liver tumor	[143]
mycb (Zebrafish)	Mifepristone	Small, typical, hypervascular and ascites of liver tumor	[143]
orf A (Human)	GAL4/UAS	Delayed onset of liver tumor	[79]
src (Zebrafish)	Constitutive	Chronic inflammation, steatosis, bile duct dilation, hyperplasia, dysplasia and HCC	[70]
src + *p53M214* (Zebrafish)	Constitutive	Steatosis, hyperplasia, dysplasia and HCC	[70]
UHRF1 (Human)	Constitutive	Atypical cells, dysplastic foci and HCC	[124]
UHRF1 + *p53M214* (Human)	Constitutive	Atypical cells, dysplastic foci and HCC	[124]
xmrk (Xiphophorus)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[80]
yy1 (Zebrafish)	Constitutive	Steatosis	[131]
zfBLP1 (Zebrafish)	Constitutive	Hyperplasia	[133]
zfMcl-1α (Zebrafish)	Constitutive	Hyperplasia	[133]

HCC: Hepatocellular carcinoma; HBx: Hepatitis B virus X protein; HCV: Hepatitis C virus.

69% of *kras-V12* transgenic larvae, respectively. Results furthermore demonstrated that blocking two pathways in *kras-V12* transgenic larvae resulted in a more significant anti-tumor effect (78%-96%)[81]. Recently, liver tumors were induced in doxycycline regulated *xmrk* transgenic fish with 100% penetration in both juveniles and adults. Overexpression of *xmrk* activated downstream targets of MEK1/2 and STAT5, which led to increased cell proliferation during tumor progression and enhanced apoptosis during tumor regression. Juvenile fish were also exposed to MEK1/2 inhibitor PD98059 or STAT5 inhibitor nicotinohydrazide in combination with doxycycline. After three weeks of treatment, abdomens and livers in 100% of transgenic fish exposed to either inhibitor were reduced relative to untreated transgenics[79]. Transient expression of the HCV core protein under the control of a CMV promoter, human hepatic lipase promoter, and zebrafish L-FABP enhancer in zebrafish embryos was used as a possible model to examine HCV replication and treatment with drugs. The amplified sub-replicon was evidence of high expression of HCV core RNA and protein. This model was used to evaluate efficacy of four HCV clinical drugs: oxymatrine, ribavirin, IFNa-2b, and vitamin B12. Vitamin B12 inhibited HCV core mRNA and protein levels in a dose-dependent manner. Ribavirin and oxymatrine drugs also significantly inhibited replication of the HCV sub-replicon. Such models may provide a novel strategy for studying mechanisms of HCV replication as well as facilitate the discovery of new anti-HCV drugs[151-153].

ZEBRAFISH MODELS FOR STUDYING DRUG-INDUCED TOXIC LIVER INJURY

Drug-induced liver injury (DILI) is a major problem in clinical pharmacology. Here, zebrafish is also promising as an animal model[25]. Zebrafish is a high-throughput *in vivo* model that can be potentially used to predict which therapeutic compounds will cause DILI in humans as well as present new markers and molecular mediators of DILI. One of the most important features of the model is that drug metabolization in zebrafish is mediated through similar pathways utilized in humans[154]. Different methods have been used in order to evaluate and quantify DILI in zebrafish. Although higher vertebrate organisms that are physiologically similar to humans have typically been used to assess DILI, the zebrafish has

similar molecular and cellular processes that accurately simulate human physiology. Therefore, zebrafish provide a significant advantage for research purposes compared to higher vertebrate organisms (*e.g.*, mice and rats). For example, the ability to assess liver damage with visually evaluable phenotypic endpoints enables the transparent larval zebrafish to be used in high-throughput screening[155,156]. In addition, DILI in embryonic or adult zebrafish exhibits histological changes, such as steatosis, apoptosis, and necrosis, that parallel human liver pathologies[157]. TAA has been shown to induce steatohepatitis in zebrafish, which is accompanied by the accumulation of fatty droplets and apoptosis[158]. AFB1 induced hepatitis and steatosis in zebrafish[133]. Zebrafish exposed to ethanol also exhibited histological changes such as steatosis, as found in alcoholic liver disease in humans[159]. Serum biochemical values, such as total bilirubin concentration and serum alanine transaminase (ALT) activity, have been determined in zebrafish[160]. Such values can be therefore used to evaluate liver function in response to drug treatment. ALT activity was found to be increased in zebrafish treated with paracetamol in a dose and time dependent fashion[157]. Furthermore, the circulating concentration of miR-122, a new experimental biomarker for liver toxicity, was increased in fish with paracetamol-induced liver injury[161].

Although many studies clearly illustrate the potential advantages of zebrafish as a model for liver toxicity, a number of challenges still exist. For example, zebrafish are exposed to a drug simply by introducing it into the water[162]. Immersion in the drug enables easy and fast administration, but the amount actually consumed by the fish is a variable even though the concentration is known and equal for all fish[163]. To overcome the problem of absorption, the quantity of the drug taken up by the fish can be determined by using a radio-labeled compound and liquid scintillation counting[154].

Before the zebrafish model can be more broadly applied, translatability of the model to humans must be confirmed. First, tests need to be conducted on established human hepatotoxic and nonhepatotoxic compounds, comparing dose responses between fish and humans. Second, translational biomarkers that bridge the gap between fish and humans must also be developed. Finally, immunological response in zebrafish must be evaluated in order to establish whether DILI develops similarly as in humans. The use of zebrafish as a model for liver injury shows promise and may enable better decision making in the early stages of drug discovery, before a compound is tested in higher mammals.

CONCLUSION

HCC is a primary malignant tumor of the liver. It is a complex disease that is accompanied by an overall poor prognosis. Although numerous oncogenes, tumor suppressor genes, and point mutations have associated with development of the disease over the past several decades, treatment options remain limited. One of the more intriguing approaches to the study of HCC and potential treatments, has been through the development of HCC disease models in zebrafish. Several zebrafish HCC models have been established through expression of various transgenes, including HBx, HCV, *myc*, *kras-G12V*, *rhoA*, *xmrk*, *src*, *edn1*, *myca*, *mycb*, or *UHRF1*. Zebrafish models have also been used for evaluation of DILI and tumor xenotransplantation. Recently, new genome editing technologies, including ZFNs, TALENs, and CRISPR/Cas systems, have been developed to facilitate targeted gene disruption in zebrafish. Together with transgenic technology, several inducible expression systems are also available for zebrafish, which will help to accelerate further development of fish models for HCC. Although establishment of liver disease and HCC models in zebrafish has led to further understanding of the molecular mechanisms and biology of these diseases, zebrafish perhaps more importantly serve as *in vivo* models with high throughput screening capabilities for the discovery of novel therapeutic agents. Novel inhibitors of angiogenesis, IRO and EM011, have been identified through such screening technology. As the utility of zebrafish for the study of HCC becomes more universally accepted, we will perhaps facilitate drug discovery and thus one day advance our treatment and the prognosis of HCC patients.

REFERENCES

1 **Stewart BW**, Wild CP, editors. World cancer report 2014. Lyon: IARC Press, 2014

2 **Bosch FX**, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; **127**: S5-S16 [PMID: 15508102]

3 **Postic C**, Dentin R, Girard J. Role of the liver in the control of carbohydrate and lipid homeostasis. *Diabetes Metab* 2004; **30**: 398-408 [PMID: 15671906]

4 **Hewitt NJ**, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 2007; **39**: 159-234 [PMID: 17364884 DOI: 10.1080/03602530601093489]

5 **Häussinger D**, Lamers WH, Moorman AF. Hepatocyte heterogeneity in the metabolism of amino acids and ammonia. *Enzyme* 1992; **46**: 72-93 [PMID: 1289083]

6 **Liu Y**, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ Health Perspect* 2010; **118**: 818-824 [PMID: 20172840 DOI: 10.1289/ehp.0901388]

7 **Baffy G**, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J Hepatol* 2012; **56**: 1384-1391 [PMID: 22326465 DOI: 10.1016/j.jhep.2011.10.027]

8 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538 [PMID: 16879891 DOI: 10.1016/j.jhep.2006.05.013]

9 **Laurent-Puig P**, Zucman-Rossi J. Genetics of hepatocellular tumors. *Oncogene* 2006; **25**: 3778-3786 [PMID: 16799619 DOI: 10.1038/sj.onc.1209547]

10 **Yang P**, Markowitz GJ, Wang XF. The hepatitis B virus-associated tumor microenvironment in hepatocellular carcinoma. *Natl Sci Rev* 2014; **1**: 396-412 [PMID: 25741453 DOI: 10.1093/nsr/nwu038]

11 **Xie N**, Chen X, Zhang T, Liu B, Huang C. Using proteomics to identify the HBx interactome in hepatitis B virus: how can this inform the clinic? *Expert Rev Proteomics* 2014; **11**: 59-74 [PMID: 24308553 DOI: 10.1586/14789450.2014.861745]

12 **Li CH**, Xu F, Chow S, Feng L, Yin D, Ng TB, Chen Y. Hepatitis B virus X protein promotes hepatocellular carcinoma transformation through interleukin-6 activation of microRNA-21 expression. *Eur J Cancer* 2014; **50**: 2560-2569 [PMID: 25087183 DOI: 10.1016/j.ejca.2014.07.008]

13 **Koike K**, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T, Kurokawa K. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *Hepatology* 1994; **19**: 810-819 [PMID: 8138251]

14 **Wurmbach E**, Chen YB, Khitrov G, Zhang W, Roayaie S, Schwartz M, Fiel I, Thung S, Mazzaferro V, Bruix J, Bottinger E, Friedman S, Waxman S, Llovet JM. Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma. *Hepatology* 2007; **45**: 938-947 [PMID: 17393520 DOI: 10.1002/hep.21622]

15 **Levrero M**. Viral hepatitis and liver cancer: the case of hepatitis C. *Oncogene* 2006; **25**: 3834-3847 [PMID: 16799625 DOI: 10.1038/sj.onc.1209562]

16 **Chen SL**, Morgan TR. The natural history of hepatitis C virus (HCV) infection. *Int J Med Sci* 2006; **3**: 47-52 [PMID: 16614742]

17 **Plumlee CR**, Lazaro CA, Fausto N, Polyak SJ. Effect of ethanol on innate antiviral pathways and HCV replication in human liver cells. *Virol J* 2005; **2**: 89 [PMID: 16324217 DOI: 10.1186/1743-422X-2-89]

18 **Voigt MD**. Alcohol in hepatocellular cancer. *Clin Liver Dis* 2005; **9**: 151-169 [PMID: 15763234 DOI: 10.1016/j.cld.2004.10.003]

19 **Siegel AB**, Zhu AX. Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. *Cancer* 2009; **115**: 5651-5661 [PMID: 19834957 DOI: 10.1002/cncr.24687]

20 **Hamid AS**, Tesfamariam IG, Zhang Y, Zhang ZG. Aflatoxin B1-induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention. *Oncol Lett* 2013; **5**: 1087-1092 [PMID: 23599745 DOI: 10.3892/ol.2013.1169]

21 **Chakraborty C**, Hsu CH, Wen ZH, Lin CS, Agoramoorthy G. Zebrafish: a complete animal model for in vivo drug discovery and development. *Curr Drug Metab* 2009; **10**: 116-124 [PMID: 19275547]

22 **McGrath P**, Li CQ. Zebrafish: a predictive model for assessing drug-induced toxicity. *Drug Discov Today* 2008; **13**: 394-401 [PMID: 18468556 DOI: 10.1016/j.drudis.2008.03.002]

23 **Dooley K**, Zon LI. Zebrafish: a model system for the study of human disease. *Curr Opin Genet Dev* 2000; **10**: 252-256 [PMID: 10826982]

24 **Santoriello C**, Zon LI. Hooked! Modeling human disease in zebrafish. *J Clin Invest* 2012; **122**: 2337-2343 [PMID: 22751109 DOI: 10.1172/JCI60434]

25 **Lieschke GJ**, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007; **8**: 353-367 [PMID: 17440532 DOI: 10.1038/nrg2091]

26 **Parng C**, Seng WL, Semino C, McGrath P. Zebrafish: a preclinical model for drug screening. *Assay Drug Dev Technol* 2002; **1**: 41-48 [PMID: 15090155 DOI: 10.1089/154065802761001293]

27 **Lu JW**, Hsia Y, Tu HC, Hsiao YC, Yang WY, Wang HD, Yuh CH. Liver development and cancer formation in zebrafish. *Birth Defects Res C Embryo Today* 2011; **93**: 157-172 [PMID: 21671355 DOI: 10.1002/bdrc.20205]

28 **Chu J**, Sadler KC. New school in liver development: lessons from zebrafish. *Hepatology* 2009; **50**: 1656-1663 [PMID: 19693947 DOI: 10.1002/hep.23157]

29 **Rekha RD**, Amali AA, Her GM, Yeh YH, Gong HY, Hu SY, Lin GH, Wu JL. Thioacetamide accelerates steatohepatitis, cirrhosis and HCC by expressing HCV core protein in transgenic zebrafish Danio rerio. *Toxicology* 2008; **243**: 11-22 [PMID: 17997003 DOI: 10.1016/j.tox.2007.09.007]

30 **Gaj T**, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; **31**: 397-405 [PMID: 23664777 DOI: 10.1016/j.tibtech.2013.04.004]

31 **Herszényi L**, Tulassay Z. Epidemiology of gastrointestinal and liver tumors. *Eur Rev Med Pharmacol Sci* 2010; **14**: 249-258 [PMID: 20496531]

32 **Lemaigre F**, Zaret KS. Liver development update: new embryo models, cell lineage control, and morphogenesis. *Curr Opin Genet Dev* 2004; **14**: 582-590 [PMID: 15380251 DOI: 10.1016/j.gde.2004.08.004]

33 **Si-Tayeb K**, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. *Dev Cell* 2010; **18**: 175-189 [PMID: 20159590 DOI: 10.1016/j.devcel.2010.01.011]

34 **Pack M**, Solnica-Krezel L, Malicki J, Neuhauss SC, Schier AF, Stemple DL, Driever W, Fishman MC. Mutations affecting development of zebrafish digestive organs. *Development* 1996; **123**: 321-328 [PMID: 9007252]

35 **Menke AL**, Spitsbergen JM, Wolterbeek AP, Woutersen RA. Normal anatomy and histology of the adult zebrafish. *Toxicol Pathol* 2011; **39**: 759-775 [PMID: 21636695 DOI: 10.1177/0192623311409597]

36 **Field HA**, Ober EA, Roeser T, Stainier DY. Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol* 2003; **253**: 279-290 [PMID: 12645931]

37 **Tao T**, Peng J. Liver development in zebrafish (Danio rerio). *J Genet Genomics* 2009; **36**: 325-334 [PMID: 19539242 DOI: 10.1016/S1673-8527(08)60121-6]

38 **Korzh S**, Pan X, Garcia-Lecea M, Winata CL, Pan X, Wohland T, Korzh V, Gong Z. Requirement of vasculogenesis and blood circulation in late stages of liver growth in zebrafish. *BMC Dev Biol* 2008; **8**: 84 [PMID: 18796162 DOI: 10.1186/1471-213X-8-84]

39 **Jung J**, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* 1999; **284**: 1998-2003 [PMID: 10373120]

40 **Niu X**, Shi H, Peng J. The role of mesodermal signals during liver organogenesis in zebrafish. *Sci China Life Sci* 2010; **53**: 455-461 [PMID: 20596911 DOI: 10.1007/s11427-010-0078-4]

41 **Shin D**, Shin CH, Tucker J, Ober EA, Rentzsch F, Poss KD, Hammerschmidt M, Mullins MC, Stainier DY. Bmp and Fgf signaling are essential for liver specification in zebrafish. *Development* 2007; **134**: 2041-2050 [PMID: 17507405 DOI: 10.1242/dev.000281]

42 **Goessling W**, North TE, Lord AM, Ceol C, Lee S, Weidinger G, Bourque C, Strijbosch R, Haramis AP, Puder M, Clevers H, Moon RT, Zon LI. APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development. *Dev Biol* 2008; **320**: 161-174 [PMID: 18585699 DOI: 10.1016/j.ydbio.2008.05.526]

43 **Wallace KN**, Yusuff S, Sonntag JM, Chin AJ, Pack M. Zebrafish hhex regulates liver development and digestive organ chirality. *Genesis* 2001; **30**: 141-143 [PMID: 11477693 DOI: 10.1002/gene.1050]

44 **Ober EA**, Field HA, Stainier DY. From endoderm formation to liver and pancreas development in zebrafish. *Mech Dev* 2003; **120**: 5-18 [PMID: 12490292]

45 **Liao W**, Ho CY, Yan YL, Postlethwait J, Stainier DY. Hhex and scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development* 2000; **127**: 4303-4313 [PMID: 11003831]

46 **Cheng W**, Guo L, Zhang Z, Soo HM, Wen C, Wu W, Peng J. HNF factors form a network to regulate liver-enriched genes in zebrafish. *Dev Biol* 2006; **294**: 482-496 [PMID: 16631158 DOI: 10.1016/j.ydbio.2006.03.018]

47 **Alexander J**, Stainier DY. A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol* 1999; **9**: 1147-1157

[PMID: 10531029 DOI: 10.1016/S0960-9822(00)80016-0]
48 **Reiter JF**, Kikuchi Y, Stainier DY. Multiple roles for Gata5 in zebrafish endoderm formation. *Development* 2001; **128**: 125-135 [PMID: 11092818]
49 **Chang C**, Hu M, Zhu Z, Lo LJ, Chen J, Peng J. liver-enriched gene 1a and 1b encode novel secretory proteins essential for normal liver development in zebrafish. *PLoS One* 2011; **6**: e22910 [PMID: 21857963 DOI: 10.1371/journal.pone.0022910]
50 **Lin TY**, Chou CF, Chung HY, Chiang CY, Li CH, Wu JL, Lin HJ, Pai TW, Hu CH, Tzou WS. Hypoxia-inducible factor 2 alpha is essential for hepatic outgrowth and functions via the regulation of leg1 transcription in the zebrafish embryo. *PLoS One* 2014; **9**: e101980 [PMID: 25000307 DOI: 10.1371/journal.pone.0101980]
51 **Noël ES**, Casal-Sueiro A, Busch-Nentwich E, Verkade H, Dong PD, Stemple DL, Ober EA. Organ-specific requirements for Hdac1 in liver and pancreas formation. *Dev Biol* 2008; **322**: 237-250 [PMID: 18687323 DOI: 10.1016/j.ydbio.2008.06.040]
52 **Farooq M**, Sulochana KN, Pan X, To J, Sheng D, Gong Z, Ge R. Histone deacetylase 3 (hdac3) is specifically required for liver development in zebrafish. *Dev Biol* 2008; **317**: 336-353 [PMID: 18367159 DOI: 10.1016/j.ydbio.2008.02.034]
53 **Sadler KC**, Krahn KN, Gaur NA, Ukomadu C. Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. *Proc Natl Acad Sci USA* 2007; **104**: 1570-1575 [PMID: 17242348 DOI: 10.1073/pnas.0610774104]
54 **Culp P**, Nüsslein-Volhard C, Hopkins N. High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc Natl Acad Sci USA* 1991; **88**: 7953-7957 [PMID: 1910170]
55 **Stuart GW**, McMurray JV, Westerfield M. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* 1988; **103**: 403-412 [PMID: 2852096]
56 **Deiters A**, Yoder JA. Conditional transgene and gene targeting methodologies in zebrafish. *Zebrafish* 2006; **3**: 415-429 [PMID: 18377222 DOI: 10.1089/zeb.2006.3.415]
57 **Stuart GW**, Vielkind JR, McMurray JV, Westerfield M. Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development* 1990; **109**: 577-584 [PMID: 2401211]
58 **Thermes V**, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly JS. I-SceI meganuclease mediates highly efficient transgenesis in fish. *Mech Dev* 2002; **118**: 91-98 [PMID: 12351173]
59 **Ivics Z**, Hackett PB, Plasterk RH, Izsvák Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* 1997; **91**: 501-510 [PMID: 9390559]
60 **Emelyanov A**, Gao Y, Naqvi NI, Parinov S. Trans-kingdom transposition of the maize dissociation element. *Genetics* 2006; **174**: 1095-1104 [PMID: 16951067 DOI: 10.1534/genetics.106.061184]
61 **Kawakami K**, Shima A, Kawakami N. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proc Natl Acad Sci USA* 2000; **97**: 11403-11408 [PMID: 11027340 DOI: 10.1073/pnas.97.21.11403]
62 **Kawakami K**, Shima A. Identification of the Tol2 transposase of the medaka fish Oryzias latipes that catalyzes excision of a nonautonomous Tol2 element in zebrafish Danio rerio. *Gene* 1999; **240**: 239-244 [PMID: 10564832]
63 **Balciunas D**, Wangensteen KJ, Wilber A, Bell J, Geurts A, Sivasubbu S, Wang X, Hackett PB, Largaespada DA, McIvor RS, Ekker SC. Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. *PLoS Genet* 2006; **2**: e169 [PMID: 17096595 DOI: 10.1371/journal.pgen.0020169]
64 **Villefranc JA**, Amigo J, Lawson ND. Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev Dyn* 2007; **236**: 3077-3087 [PMID: 17948311 DOI: 10.1002/dvdy.21354]
65 **Kwan KM**, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* 2007; **236**: 3088-3099 [PMID: 17937395 DOI: 10.1002/dvdy.21343]
66 **Walhout AJ**, Temple GF, Brasch MA, Hartley JL, Lorson MA, van den Heuvel S, Vidal M. GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol* 2000; **328**: 575-592 [PMID: 11075367]
67 **Her GM**, Yeh YH, Wu JL. 435-bp liver regulatory sequence in the liver fatty acid binding protein (L-FABP) gene is sufficient to modulate liver regional expression in transgenic zebrafish. *Dev Dyn* 2003; **227**: 347-356 [PMID: 12815620 DOI: 10.1002/dvdy.10324]
68 **Her GM**, Chiang CC, Chen WY, Wu JL. In vivo studies of liver-type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (Danio rerio). *FEBS Lett* 2003; **538**: 125-133 [PMID: 12633865]
69 **Lu JW**, Liao CY, Yang WY, Lin YM, Jin SL, Wang HD, Yuh CH. Overexpression of endothelin 1 triggers hepatocarcinogenesis in zebrafish and promotes cell proliferation and migration through the AKT pathway. *PLoS One* 2014; **9**: e85318 [PMID: 24416389 DOI: 10.1371/journal.pone.0085318]
70 **Lu JW**, Yang WY, Tsai SM, Lin YM, Chang PH, Chen JR, Wang HD, Wu JL, Jin SL, Yuh CH. Liver-specific expressions of HBx and src in the p53 mutant trigger hepatocarcinogenesis in zebrafish. *PLoS One* 2013; **8**: e76951 [PMID: 24130815 DOI: 10.1371/journal.pone.0076951]
71 **Huang X**, Nguyen AT, Li Z, Emelyanov A, Parinov S, Gong Z. One step forward: the use of transgenic zebrafish tumor model in drug screens. *Birth Defects Res C Embryo Today* 2011; **93**: 173-181 [PMID: 21671356 DOI: 10.1002/bdrc.20208]
72 **Blechinger SR**, Evans TG, Tang PT, Kuwada JY, Warren JT, Krone PH. The heat-inducible zebrafish hsp70 gene is expressed during normal lens development under non-stress conditions. *Mech Dev* 2002; **112**: 213-215 [PMID: 11850198]
73 **Shoji W**, Sato-Maeda M. Application of heat shock promoter in transgenic zebrafish. *Dev Growth Differ* 2008; **50**: 401-406 [PMID: 18430027 DOI: 10.1111/j.1440-169X.2008.01038.x]
74 **Yang YS**, Hughes TE. Cre stoplight: a red/green fluorescent reporter of Cre recombinase expression in living cells. *Biotechniques* 2001; **31**: 1036, 1038, 1040-1041 [PMID: 11730010]
75 **Seok SH**, Na YR, Han JH, Kim TH, Jung H, Lee BH, Emelyanov A, Parinov S, Park JH. Cre/loxP-regulated transgenic zebrafish model for neural progenitor-specific oncogenic Kras expression. *Cancer Sci* 2010; **101**: 149-154 [PMID: 19961491 DOI: 10.1111/j.1349-7006.2009.01393.x]
76 **Fischer JA**, Giniger E, Maniatis T, Ptashne M. GAL4 activates transcription in Drosophila. *Nature* 1988; **332**: 853-856 [PMID: 3128741 DOI: 10.1038/332853a0]
77 **Brand AH**, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 1993; **118**: 401-415 [PMID: 8223268]
78 **Scheer N**, Campos-Ortega JA. Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. *Mech Dev* 1999; **80**: 153-158 [PMID: 10072782]
79 **Zhan H**, Spitsbergen JM, Qing W, Wu YL, Paul TA, Casey JW, Her GM, Gong Z. Transgenic expression of walleye dermal sarcoma virus rv-cyclin gene in zebrafish and its suppressive effect on liver tumor development after carcinogen treatment. *Mar Biotechnol* (NY) 2010; **12**: 640-649 [PMID: 20052603 DOI: 10.1007/s10126-009-9251-9]
80 **Li Z**, Huang X, Zhan H, Zeng Z, Li C, Spitsbergen JM, Meierjohann S, Schartl M, Gong Z. Inducible and repressable oncogene-addicted hepatocellular carcinoma in Tet-on xmrk transgenic zebrafish. *J Hepatol* 2012; **56**: 419-425 [PMID: 21888874 DOI: 10.1016/j.jhep.2011.07.025]
81 **Liu W**, Chen JR, Hsu CH, Li YH, Chen YM, Lin CY, Huang SJ, Chang ZK, Chen YC, Lin CH, Gong HY, Lin CC, Kawakami K, Wu JL. A zebrafish model of intrahepatic cholangiocarcinoma by dual expression of hepatitis B virus X and hepatitis C virus core protein in liver. *Hepatology* 2012; **56**: 2268-2276 [PMID:

22729936 DOI: 10.1002/hep.25914]
82 **Nguyen AT**, Emelyanov A, Koh CH, Spitsbergen JM, Parinov S, Gong Z. An inducible kras(V12) transgenic zebrafish model for liver tumorigenesis and chemical drug screening. *Dis Model Mech* 2012; **5**: 63-72 [PMID: 21903676 DOI: 10.1242/dmm.008367]
83 **Li Z**, Zheng W, Wang Z, Zeng Z, Zhan H, Li C, Zhou L, Yan C, Spitsbergen JM, Gong Z. A transgenic zebrafish liver tumor model with inducible Myc expression reveals conserved Myc signatures with mammalian liver tumors. *Dis Model Mech* 2013; **6**: 414-423 [PMID: 23038063 DOI: 10.1242/dmm.010462]
84 **Meng X**, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol* 2008; **26**: 695-701 [PMID: 18500337 DOI: 10.1038/nbt1398]
85 **Huang P**, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol* 2011; **29**: 699-700 [PMID: 21822242 DOI: 10.1038/nbt.1939]
86 **Hwang WY**, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 227-229 [PMID: 23360964 DOI: 10.1038/nbt.2501]
87 **Kim H**, Kim JS. A guide to genome engineering with programmable nucleases. *Nat Rev Genet* 2014; **15**: 321-334 [PMID: 24690881 DOI: 10.1038/nrg3686]
88 **Ul Ain Q**, Chung JY, Kim YH. Current and future delivery systems for engineered nucleases: ZFN, TALEN and RGEN. *J Control Release* 2015; **205**: 120-127 [PMID: 25553825 DOI: 10.1016/j.jconrel.2014.12.036]
89 **Bitinaite J**, Wah DA, Aggarwal AK, Schildkraut I. FokI dimerization is required for DNA cleavage. *Proc Natl Acad Sci USA* 1998; **95**: 10570-10575 [PMID: 9724744]
90 **Urnov FD**, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010; **11**: 636-646 [PMID: 20717154 DOI: 10.1038/nrg2842]
91 **Miller J**, McLachlan AD, Klug A. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J* 1985; **4**: 1609-1614 [PMID: 4040853]
92 **Vanamee ES**, Santagata S, Aggarwal AK. FokI requires two specific DNA sites for cleavage. *J Mol Biol* 2001; **309**: 69-78 [PMID: 11491302 DOI: 10.1006/jmbi.2001.4635]
93 **Gupta RM**, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *J Clin Invest* 2014; **124**: 4154-4161 [PMID: 25271723 DOI: 10.1172/JCI72992]
94 **Segal DJ**, Beerli RR, Blancafort P, Dreier B, Effertz K, Huber A, Koksch B, Lund CV, Magnenat L, Valente D, Barbas CF. Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry* 2003; **42**: 2137-2148 [PMID: 12590603 DOI: 10.1021/bi026806o]
95 **Maeder ML**, Thibodeau-Beganny S, Sander JD, Voytas DF, Joung JK. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat Protoc* 2009; **4**: 1471-1501 [PMID: 19798082 DOI: 10.1038/nprot.2009.98]
96 **Sander JD**, Dahlborg EJ, Goodwin MJ, Cade L, Zhang F, Cifuentes D, Curtin SJ, Blackburn JS, Thibodeau-Beganny S, Qi Y, Pierick CJ, Hoffman E, Maeder ML, Khayter C, Reyon D, Dobbs D, Langenau DM, Stupar RM, Giraldez AJ, Voytas DF, Peterson RT, Yeh JR, Joung JK. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods* 2011; **8**: 67-69 [PMID: 21151135 DOI: 10.1038/nmeth.1542]
97 **Hockemeyer D**, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol* 2009; **27**: 851-857 [PMID: 19680244 DOI: 10.1038/nbt.1562]
98 **Miller JC**, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol* 2007; **25**: 778-785 [PMID: 17603475 DOI: 10.1038/nbt1319]
99 **Szczepek M**, Brondani V, Büchel J, Serrano L, Segal DJ, Cathomen T. Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol* 2007; **25**: 786-793 [PMID: 17603476 DOI: 10.1038/nbt1317]
100 **Doyon Y**, Vo TD, Mendel MC, Greenberg SG, Wang J, Xia DF, Miller JC, Urnov FD, Gregory PD, Holmes MC. Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nat Methods* 2011; **8**: 74-79 [PMID: 21131970 DOI: 10.1038/nmeth.1539]
101 **Li T**, Liu B, Spalding MH, Weeks DP, Yang B. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat Biotechnol* 2012; **30**: 390-392 [PMID: 22565958 DOI: 10.1038/nbt.2199]
102 **Joung JK**, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* 2013; **14**: 49-55 [PMID: 23169466 DOI: 10.1038/nrm3486]
103 **Deng D**, Yan C, Pan X, Mahfouz M, Wang J, Zhu JK, Shi Y, Yan N. Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 2012; **335**: 720-723 [PMID: 22223738 DOI: 10.1126/science.1215670]
104 **Bedell VM**, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG, Tan W, Penheiter SG, Ma AC, Leung AY, Fahrenkrug SC, Carlson DF, Voytas DF, Clark KJ, Essner JJ, Ekker SC. In vivo genome editing using a high-efficiency TALEN system. *Nature* 2012; **491**: 114-118 [PMID: 23000899 DOI: 10.1038/nature11537]
105 **Reyon D**, Khayter C, Regan MR, Joung JK, Sander JD. Engineering designer transcription activator-like effector nucleases (TALENs) by REAL or REAL-Fast assembly. *Curr Protoc Mol Biol* 2012; **Chapter 12**: Unit 12.15 [PMID: 23026907 DOI: 10.1002/0471142727.mb1215s100]
106 **Weber E**, Gruetzner R, Werner S, Engler C, Marillonnet S. Assembly of designer TAL effectors by Golden Gate cloning. *PLoS One* 2011; **6**: e19722 [PMID: 21625552 DOI: 10.1371/journal.pone.0019722]
107 **Reyon D**, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* 2012; **30**: 460-465 [PMID: 22484455 DOI: 10.1038/nbt.2170]
108 **Wiedenheft B**, Sternberg SH, Doudna JA. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 2012; **482**: 331-338 [PMID: 22337052 DOI: 10.1038/nature10886]
109 **Deltcheva E**, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011; **471**: 602-607 [PMID: 21455174 DOI: 10.1038/nature09886]
110 **Ding Q**, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell* 2013; **12**: 393-394 [PMID: 23561441 DOI: 10.1016/j.stem.2013.03.006]
111 **Ablain J**, Durand EM, Yang S, Zhou Y, Zon LI. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev Cell* 2015; **32**: 756-764 [PMID: 25752963 DOI: 10.1016/j.devcel.2015.01.032]
112 **Kim CM**, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* 1991; **351**: 317-320 [PMID: 2034275 DOI: 10.1038/351317a0]
113 **Seifer M**, Höhne M, Schaefer S, Gerlich WH. In vitro tumorigenicity of hepatitis B virus DNA and HBx protein. *J Hepatol* 1991; **13** Suppl 4: S61-S65 [PMID: 1822516]
114 **Wu BK**, Li CC, Chen HJ, Chang JL, Jeng KS, Chou CK, Hsu MT, Tsai TF. Blocking of G1/S transition and cell death in the regenerating liver of Hepatitis B virus X protein transgenic mice. *Biochem Biophys Res Commun* 2006; **340**: 916-928 [PMID: 16403455 DOI: 10.1016/j.bbrc.2005.12.089]
115 **Jeannot E**, Boorman GA, Kosyk O, Bradford BU, Shymoniak S, Tumurbaatar B, Weinman SA, Melnyk SB, Tryndyak V, Pogribny

IP, Rusyn I. Increased incidence of aflatoxin B1-induced liver tumors in hepatitis virus C transgenic mice. *Int J Cancer* 2012; **130**: 1347-1356 [PMID: 21500192 DOI: 10.1002/ijc.26140]
116 **Lian M**, Liu Y, Yu SZ, Qian GS, Wan SG, Dixon KR. Hepatitis B virus x gene and cyanobacterial toxins promote aflatoxin B1-induced hepatotumorigenesis in mice. *World J Gastroenterol* 2006; **12**: 3065-3072 [PMID: 16718789]
117 **Akriviadis EA**, Llovet JM, Efremidis SC, Shouval D, Canelo R, Ringe B, Meyers WC. Hepatocellular carcinoma. *Br J Surg* 1998; **85**: 1319-1331 [PMID: 9782009 DOI: 10.1046/j.1365-2168.1998.00865.x]
118 **Aguilar F**, Harris CC, Sun T, Hollstein M, Cerutti P. Geographic variation of p53 mutational profile in nonmalignant human liver. *Science* 1994; **264**: 1317-1319 [PMID: 8191284]
119 **Pang R**, Tse E, Poon RT. Molecular pathways in hepatocellular carcinoma. *Cancer Lett* 2006; **240**: 157-169 [PMID: 16239065 DOI: 10.1016/j.canlet.2005.08.031]
120 **Shieh YS**, Chang YS, Hong JR, Chen LJ, Jou LK, Hsu CC, Her GM. Increase of hepatic fat accumulation by liver specific expression of Hepatitis B virus X protein in zebrafish. *Biochim Biophys Acta* 2010; **1801**: 721-730 [PMID: 20416398 DOI: 10.1016/j.bbalip.2010.04.008]
121 **Chen LJ**, Hsu CC, Hong JR, Jou LK, Tseng HC, Wu JL, Liou YC, Her GM. Liver-specific expression of p53-negative regulator mdm2 leads to growth retardation and fragile liver in zebrafish. *Dev Dyn* 2008; **237**: 1070-1081 [PMID: 18297734 DOI: 10.1002/dvdy.21477]
122 **Higashitsuji H**, Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo Y, Masuda T, Dawson S, Shimada Y, Mayer RJ, Fujita J. The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53. *Cancer Cell* 2005; **8**: 75-87 [PMID: 16023600 DOI: 10.1016/j.ccr.2005.06.006]
123 **Her GM**, Hsu CC, Hong JR, Lai CY, Hsu MC, Pang HW, Chan SK, Pai WY. Overexpression of gankyrin induces liver steatosis in zebrafish (Danio rerio). *Biochim Biophys Acta* 2011; **1811**: 536-548 [PMID: 21722753 DOI: 10.1016/j.bbalip.2011.06.011]
124 **Mudbhary R**, Hoshida Y, Chernyavskaya Y, Jacob V, Villanueva A, Fiel MI, Chen X, Kojima K, Thung S, Bronson RT, Lachenmayer A, Revill K, Alsinet C, Sachidanandam R, Desai A, SenBanerjee S, Ukomadu C, Llovet JM, Sadler KC. UHRF1 overexpression drives DNA hypomethylation and hepatocellular carcinoma. *Cancer Cell* 2014; **25**: 196-209 [PMID: 24486181 DOI: 10.1016/j.ccr.2014.01.003]
125 **LaPierre LA**, Casey JW, Holzschu DL. Walleye retroviruses associated with skin tumors and hyperplasias encode cyclin D homologs. *J Virol* 1998; **72**: 8765-8771 [PMID: 9765420]
126 **Quackenbush SL**, Holzschu DL, Bowser PR, Casey JW. Transcriptional analysis of walleye dermal sarcoma virus (WDSV). *Virology* 1997; **237**: 107-112 [PMID: 9344912 DOI: 10.1006/viro.1997.8755]
127 **Lu JW**, Hsia Y, Yang WY, Lin YI, Li CC, Tsai TF, Chang KW, Shieh GS, Tsai SF, Wang HD, Yuh CH. Identification of the common regulators for hepatocellular carcinoma induced by hepatitis B virus X antigen in a mouse model. *Carcinogenesis* 2012; **33**: 209-219 [PMID: 22021908 DOI: 10.1093/carcin/bgr224]
128 **Zhang L**, Cai X, Chen K, Wang Z, Wang L, Ren M, Huang A, Tang H. Hepatitis B virus protein up-regulated HLJ1 expression via the transcription factor YY1 in human hepatocarcinoma cells. *Virus Res* 2011; **157**: 76-81 [PMID: 21345358 DOI: 10.1016/j.virusres.2011.02.009]
129 **Sung WK**, Lu Y, Lee CW, Zhang D, Ronaghi M, Lee CG. Deregulated direct targets of the hepatitis B virus (HBV) protein, HBx, identified through chromatin immunoprecipitation and expression microarray profiling. *J Biol Chem* 2009; **284**: 21941-21954 [PMID: 19439406 DOI: 10.1074/jbc.M109.014563]
130 **Zhang S**, Jiang T, Feng L, Sun J, Lu H, Wang Q, Pan M, Huang D, Wang X, Wang L, Jin H. Yin Yang-1 suppresses differentiation of hepatocellular carcinoma cells through the downregulation of CCAAT/enhancer-binding protein alpha. *J Mol Med* (Berl) 2012; **90**: 1069-1077 [PMID: 22391813 DOI: 10.1007/s00109-012-0879-y]
131 **Her GM**, Pai WY, Lai CY, Hsieh YW, Pang HW. Ubiquitous transcription factor YY1 promotes zebrafish liver steatosis and lipotoxicity by inhibiting CHOP-10 expression. *Biochim Biophys Acta* 2013; **1831**: 1037-1051 [PMID: 23416188 DOI: 10.1016/j.bbalip.2013.02.002]
132 **Pai WY**, Hsu CC, Lai CY, Chang TZ, Tsai YL, Her GM. Cannabinoid receptor 1 promotes hepatic lipid accumulation and lipotoxicity through the induction of SREBP-1c expression in zebrafish. *Transgenic Res* 2013; **22**: 823-838 [PMID: 23315130 DOI: 10.1007/s11248-012-9685-0]
133 **Her GM**, Cheng CH, Hong JR, Sundaram GS, Wu JL. Imbalance in liver homeostasis leading to hyperplasia by overexpressing either one of the Bcl-2-related genes, zfBLP1 and zfMcl-1a. *Dev Dyn* 2006; **235**: 515-523 [PMID: 16273521 DOI: 10.1002/dvdy.20624]
134 **Lu JW**, Yang WY, Lin YM, Jin SL, Yuh CH. Hepatitis B virus X antigen and aflatoxin B1 synergistically cause hepatitis, steatosis and liver hyperplasia in transgenic zebrafish. *Acta Histochem* 2013; **115**: 728-739 [PMID: 23499292 DOI: 10.1016/j.acthis.2013.02.012]
135 **Hayashi J**, Aoki H, Kajino K, Moriyama M, Arakawa Y, Hino O. Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor alpha. *Hepatology* 2000; **32**: 958-961 [PMID: 11050045 DOI: 10.1053/jhep.2000.19343]
136 **Nguyen AT**, Emelyanov A, Koh CH, Spitsbergen JM, Lam SH, Mathavan S, Parinov S, Gong Z. A high level of liver-specific expression of oncogenic Kras(V12) drives robust liver tumorigenesis in transgenic zebrafish. *Dis Model Mech* 2011; **4**: 801-813 [PMID: 21729876 DOI: 10.1242/dmm.007831]
137 **Chew TW**, Liu XJ, Liu L, Spitsbergen JM, Gong Z, Low BC. Crosstalk of Ras and Rho: activation of RhoA abates Kras-induced liver tumorigenesis in transgenic zebrafish models. *Oncogene* 2014; **33**: 2717-2727 [PMID: 23812423 DOI: 10.1038/onc.2013.240]
138 **Li Z**, Luo H, Li C, Huo X, Yan C, Huang X, Al-Haddawi M, Mathavan S, Gong Z. Transcriptomic analysis of a transgenic zebrafish hepatocellular carcinoma model reveals a prominent role of immune responses in tumour progression and regression. *Int J Cancer* 2014; **135**: 1564-1573 [PMID: 24550086 DOI: 10.1002/ijc.28794]
139 **Rautou PE**, Mansouri A, Lebrec D, Durand F, Valla D, Moreau R. Autophagy in liver diseases. *J Hepatol* 2010; **53**: 1123-1134 [PMID: 20810185 DOI: 10.1016/j.jhep.2010.07.006]
140 **Takamura A**, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* 2011; **25**: 795-800 [PMID: 21498569 DOI: 10.1101/gad.2016211]
141 **Cui J**, Sim TH, Gong Z, Shen HM. Generation of transgenic zebrafish with liver-specific expression of EGFP-Lc3: a new in vivo model for investigation of liver autophagy. *Biochem Biophys Res Commun* 2012; **422**: 268-273 [PMID: 22580284 DOI: 10.1016/j.bbrc.2012.04.145]
142 **Zheng W**, Li Z, Nguyen AT, Li C, Emelyanov A, Gong Z. Xmrk, kras and myc transgenic zebrafish liver cancer models share molecular signatures with subsets of human hepatocellular carcinoma. *PLoS One* 2014; **9**: e91179 [PMID: 24633177 DOI: 10.1371/journal.pone.0091179]
143 **Sun L**, Nguyen AT, Spitsbergen JM, Gong Z. Myc-induced liver tumors in transgenic zebrafish can regress in tp53 null mutation. *PLoS One* 2015; **10**: e0117249 [PMID: 25612309 DOI: 10.1371/journal.pone.0117249]
144 **Gómez del Pulgar T**, Benitah SA, Valerón PF, Espina C, Lacal JC. Rho GTPase expression in tumourigenesis: evidence for a significant link. *Bioessays* 2005; **27**: 602-613 [PMID: 15892119 DOI: 10.1002/bies.20238]
145 **Michelini E**, Cevenini L, Mezzanotte L, Coppa A, Roda A. Cell-based assays: fuelling drug discovery. *Anal Bioanal Chem* 2010; **398**: 227-238 [PMID: 20623273 DOI: 10.1007/s00216-010-

3933-z]
146 **Xiang J**, Yang H, Che C, Zou H, Yang H, Wei Y, Quan J, Zhang H, Yang Z, Lin S. Identifying tumor cell growth inhibitors by combinatorial chemistry and zebrafish assays. *PLoS One* 2009; **4**: e4361 [PMID: 19194508 DOI: 10.1371/journal.pone.0004361]
147 **Konantz M**, Balci TB, Hartwig UF, Dellaire G, André MC, Berman JN, Lengerke C. Zebrafish xenografts as a tool for in vivo studies on human cancer. *Ann N Y Acad Sci* 2012; **1266**: 124-137 [PMID: 22901264 DOI: 10.1111/j.1749-6632.2012.06575.x]
148 **Lee SL**, Rouhi P, Dahl Jensen L, Zhang D, Ji H, Hauptmann G, Ingham P, Cao Y. Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model. *Proc Natl Acad Sci USA* 2009; **106**: 19485-19490 [PMID: 19887629 DOI: 10.1073/pnas.0909228106]
149 **Karna P**, Rida PC, Turaga RC, Gao J, Gupta M, Fritz A, Werner E, Yates C, Zhou J, Aneja R. A novel microtubule-modulating agent EM011 inhibits angiogenesis by repressing the HIF-1α axis and disrupting cell polarity and migration. *Carcinogenesis* 2012; **33**: 1769-1781 [PMID: 22678119 DOI: 10.1093/carcin/bgs200]
150 **Tran TC**, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, Baranowski TC, Rubinstein AL, Doan TN, Dingledine R, Sandberg EM. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res* 2007; **67**: 11386-11392 [PMID: 18056466 DOI: 10.1158/0008-5472.CAN-07-3126]
151 **Wang C**, Tao W, Wang Y, Bikow J, Lu B, Keating A, Verma S, Parker TG, Han R, Wen XY. Rosuvastatin, identified from a zebrafish chemical genetic screen for antiangiogenic compounds, suppresses the growth of prostate cancer. *Eur Urol* 2010; **58**: 418-426 [PMID: 20605315 DOI: 10.1016/j.eururo.2010.05.024]
152 **Ding CB**, Zhao Y, Zhang JP, Peng ZG, Song DQ, Jiang JD. A zebrafish model for subgenomic hepatitis C virus replication. *Int J Mol Med* 2015; **35**: 791-797 [PMID: 25572289 DOI: 10.3892/ijmm.2015.2063]
153 **Zhao Y**, Qin W, Zhang JP, Hu ZY, Tong JW, Ding CB, Peng ZG, Zhao LX, Song DQ, Jiang JD. HCV IRES-mediated core expression in zebrafish. *PLoS One* 2013; **8**: e56985 [PMID: 23469178 DOI: 10.1371/journal.pone.0056985]
154 **Ding CB**, Zhang JP, Zhao Y, Peng ZG, Song DQ, Jiang JD. Zebrafish as a potential model organism for drug test against hepatitis C virus. *PLoS One* 2011; **6**: e22921 [PMID: 21857967 DOI: 10.1371/journal.pone.0022921]
155 **Vliegenthart AD**, Tucker CS, Del Pozo J, Dear JW. Zebrafish as model organisms for studying drug-induced liver injury. *Br J Clin Pharmacol* 2014; **78**: 1217-1227 [PMID: 24773296 DOI: 10.1111/bcp.12408]
156 **He JH**, Guo SY, Zhu F, Zhu JJ, Chen YX, Huang CJ, Gao JM, Dong QX, Xuan YX, Li CQ. A zebrafish phenotypic assay for assessing drug-induced hepatotoxicity. *J Pharmacol Toxicol Methods* 2013; **67**: 25-32 [PMID: 23128142 DOI: 10.1016/j.vascn.2012.10.003]
157 **Hill A**, Mesens N, Steemans M, Xu JJ, Aleo MD. Comparisons between in vitro whole cell imaging and in vivo zebrafish-based approaches for identifying potential human hepatotoxicants earlier in pharmaceutical development. *Drug Metab Rev* 2012; **44**: 127-140 [PMID: 22242931 DOI: 10.3109/03602532.2011.645578]
158 **North TE**, Babu IR, Vedder LM, Lord AM, Wishnok JS, Tannenbaum SR, Zon LI, Goessling W. PGE2-regulated wnt signaling and N-acetylcysteine are synergistically hepatoprotective in zebrafish acetaminophen injury. *Proc Natl Acad Sci USA* 2010; **107**: 17315-17320 [PMID: 20855591 DOI: 10.1073/pnas.1008209107]
159 **Amali AA**, Rekha RD, Lin CJ, Wang WL, Gong HY, Her GM, Wu JL. Thioacetamide induced liver damage in zebrafish embryo as a disease model for steatohepatitis. *J Biomed Sci* 2006; **13**: 225-232 [PMID: 16456712 DOI: 10.1007/s11373-005-9055-5]
160 **Passeri MJ**, Cinaroglu A, Gao C, Sadler KC. Hepatic steatosis in response to acute alcohol exposure in zebrafish requires sterol regulatory element binding protein activation. *Hepatology* 2009; **49**: 443-452 [PMID: 19127516 DOI: 10.1002/hep.22667]
161 **Murtha JM**, Qi W, Keller ET. Hematologic and serum biochemical values for zebrafish (Danio rerio). *Comp Med* 2003; **53**: 37-41 [PMID: 12625505]
162 **Vliegenthart AD**, Starkey Lewis P, Tucker CS, Del Pozo J, Rider S, Antoine DJ, Dubost V, Westphal M, Moulin P, Bailey MA, Moggs JG, Goldring CE, Park BK, Dear JW. Retro-orbital blood acquisition facilitates circulating microRNA measurement in zebrafish with paracetamol hepatotoxicity. *Zebrafish* 2014; **11**: 219-226 [PMID: 24625211 DOI: 10.1089/zeb.2013.0912]
163 **Burns CG**, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nat Chem Biol* 2005; **1**: 263-264 [PMID: 16408054 DOI: 10.1038/nchembio732]

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