A zebrafish model for subgenomic hepatitis C virus replication

CUN-BAO DING[1,2], YE ZHAO[1], JING-PU ZHANG[1], ZONG-GEN PENG[1], DAN-QING SONG[1] and JIAN-DONG JIANG[1]

[1]Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050; [2]College of Life Sciences, Hebei United University, Tangshan, Hebei 063009, P.R. China

Received July 2, 2014; Accepted December 15, 2014

DOI: 10.3892/ijmm.2015.2063

Abstract. Persistent infection with hepatitis C virus (HCV) is a major risk factor in the development of hepatocellular carcinoma. The elucidation of the pathogenesis of HCV-associated liver disease is hampered by the absence of an appropriate small animal model. Zebrafish exhibits high genetic homology to mammals, and is easily manipulated experimentally. In this study, we describe the use of a zebrafish model for the analysis of HCV replication mechanisms. As the 5' untranslated region (UTR), the core protein, the non-structural protein 5B (NS5B) and the 3'UTR are essential for HCV replication, we constructed a HCV sub-replicon gene construct including the 4 gene sequences and the enhanced green fluorescent protein (EGFP) reporter gene; these genes were transcribed through the mouse hepatocyte nuclear factor 4 (mHNF4) promoter. By microinjection of the subgenomic replicon vector into zebrafish larvae, the virus was easily detected by observing EGFP fluorescence in the liver. The positive core and NS5B signals showed positive expression of the HCV gene construct in zebrafish by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis. Importantly, the negative strand sequence of the HCV subgenomic RNA was detected by RT-PCR and hybridization *in situ*, demonstrating that the HCV sub-replicon has positive replication activity. Furthermore, the hybridization signal mainly appeared in the liver region of larvae, as detected by the sense probe of the core protein or NS5B, which confirmed that the sub-replicon amplification occurred in the zebrafish liver. The amplification of the sub-replicon caused alterations in the expression of certain genes, which is similar to HCV infection in human liver cells. To verify the use of this zebrafish model in drug evaluation, two drugs against HCV used in clinical practice, ribavirin and oxymatrine, were tested and these drugs showed significant inhibition of replication of the HCV sub-replicon in the larvae. In conclusion, this zebrafish model of HCV may prove to be a novel and simple *in vivo* model for the study of the mechanisms of HCV replication and may also prove useful in the disovery of new anti-HCV drugs.

Correspondence to: Dr Jing-Pu Zhang or Dr Jian-Dong Jiang, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Tiantan Xili, Dongcheng, Beijing 100050, P.R. China
E-mail: zjp5577@126.com
E-mail: jiang.jdong@163.com

Abbreviations: HCV, hepatitis C virus; UTR, untranslated region; NS5B, non-structural protein 5B; mHNF4, mouse hepatocyte nuclear factor 4; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; dpf, days post-fertilization; EMCV, encephalomyocarditis virus; WT, wild-type; ACC, acetyl-CoA carboxylase; PBST, phosphate-buffered saline containing Tween-20

Key words: hepatitis C virus, sub-replicon, non-structural protein 5B, core protein, zebrafish

Introduction

Hepatitis C virus (HCV) is the cause of chronic infection in approximately 180 million individuals worldwide (1). However, the elucidation of the pathogenesis of HCV-associated liver disease is hampered by the absence of an appropriate small animal model. HCV genomic RNA can be used as either mRNA for translation or a template for RNA replication. The translation initiation of the HCV genome is controlled by an internal ribosome-entry site (IRES) within the 5' untranslated region (UTR) (2). The 3'UTR has been shown to serve as a replication onset site for recognition and nucleotide incorporation by non-structural protein 5B (NS5B), an RNA-dependent RNA polymerase (3). The NS5B polymerase is a 65-kDa protein responsible for HCV RNA replication (4) that recognizes a specific RNA sequence in the 3'UTR of the genomic RNA and replicates the primary negative strand RNA first, and then the positive-strand RNA according to the negative strand RNA template (5). It has been shown that NS5B alone can copy an RNA template containing the HCV UTR *in vitro* or *in vivo*, without the need for any other viral/host factors (6). Based on the HCV elements mentioned above (2-6), a HCV subgenomic replicon (sub-replicon) was designed in our laboratory. Zebrafish is a good model organism for human diseases, and a number of disease models have emerged from studies using this organism (7,8). There are several advantages to using zebrafish embryos to develop models of liver diseases (8-10); the zebrafish exhibits high homology to humans genetically (11) and is small and easy-to-handle experimentally. We therefore explored the potential of using zebrafish as a host for HCV replication. In our previous study, we demonstrated that zebrafish may be a model organism to host HCV (12). The HCV sub-replicon was created

with 2 vectors, one with HCV ns5b and the other containing the minus strand of the HCV 5'UTR, core and 3'UTR by co-injecting into zebrafish zygotes. The sub-replicon amplified in the liver showed a significant expression of HCV core RNA and protein. As HCV is a plus strand RNA virus, we then intended to generate a plus strand HCV sub-replicon zebrafish model for anti-HCV drug screening. In this study, we describe the construction of a HCV subgenomic expression vector using the mouse hepatocyte nuclear factor 4 (mHNF4) promoter, the HCV 5'UTR-core protein, the enhanced green fluorescent protein (EGFP) reporter gene and the NS5B 3'UTR. This study describes the use of a zebrafish model for further investigations into the mechanisms of HCV replication and the pathology of HCV infection in the liver *in vivo*. This model may also aid drug evaluation studies and may thus aid in the discovery of new anti-HCV drugs.

Materials and methods

Plasmids, reagents and antibodies. HCV strain 1b (J4L6s; Accession no. AF054247) was provided by Dr H.S. Chen (Institute of Medicinal Biotechnology, Beijing, China). The sub-replicon construct (pH5B) was constructed by the insertion of the HCV 5'UTR-core downstream of the mHNF4a promoter (the promoter sequence was cloned and identified by sequencing in our preliminary experiment) into the pIRES2-EGFP plasmid, followed by the insertion of the IRES-NS5B-3'UTR after the EGFP gene using the *Not*I and *Xba*I restriction sites (Fig. 1A). All the constructs were confirmed by DNA sequencing. The antibodies against NS5B (ab35586), the core protein (sc-8334) and GFP (ab2740) were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively.

Zebrafish microinjection and fluorescence microscopy. The adult zebrafish strain AB (*Danio rerio*) was obtained from Dr Anming Meng (Tsinghua University, Beijing, China). The zebrafish were maintained in a controlled environment under a 14-h light/10-h dark cycle at 28±1˚C.

The pH5B construct was linearized and injected into the blastomere of 1-8-cell stage embryos at 1 ng/μl. Larvae positive for GFP were examined at 8 and 12 days post-fertilization (dpf), using a fluorescence microscope (IX51; Olympus, Tokyo, Japan) with GFP filters (480 nm excitation, 505 nm emission).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the larvae using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 1 μg of the total RNA using AMV reverse transcriptase (Promega, Madison, WI, USA) with different reverse transcription primers: NS5B-R and $d(T)_{18}$ for transcript resulting from mHNF4a promoter, and core-F for HCV sub-replicon replication products. The target cDNA templates were then amplified by PCR using Taq polymerase (Takara Bio Inc., Shiga, Japan) and the primer pairs for the core protein, NS5B and β-actin are listed in Table I. The cDNA template of non-transgenic wild-type zebrafish larvae was used as a negative control. PCR was performed with 0.5 μl of cDNA under the following cycling conditions: 94˚C for 5 min, then 30 cycles of 94˚C for 60 sec, 55˚C for 30 sec and 72 ˚C for 1 min, followed by an additional extension step at 72˚C for 10 min to allow for complete synthesis. The RT-PCR products were subjected to 1.5% agarose gel electrophoresis. β-actin was used as a control.

Table I. RT-PCR primer sequences for HCV.

Core	F: 5'-AGCGGTCGCAACCTCGTGGAA-3'
Core	R: 5'-GCGGAAGCTGGGATGGTCAAAC-3'
NS5B	F: 5'-GCTCGCCTTATCGTATTCC-3'
NS5B	R: 5'-AGTCGTCAGCACGCCAC-3'
GFP	F: 5'-ACGGCGTGCAGTGCTT-3'
GFP	R: 5'-TGGGTGCTCAGGTAGTGG-3'
β-actin	F: 5'-AGGGAAATCGTGGGTGACATCAAA-3'
β-actin	R: 5'-ACTCATCGTACTCCTGCTTGCTGA-3'
Chemokine20	F: 5'-TCTCTTCTCACCTGCCCTAA-3'
Chemokine20	R: 5'-ATTGCTTGCACCTTCTCCCTC-3'
AHSG	F: 5'-GGAAGGCAGCGGTGAAA-3'
AHSG	R: 5'-ATGGTCTGGCCCGAGTG-3'
Hsp70	F: 5'-GCGACACCTCTGGAAAC-3'
Hsp70	R: 5'-TGCTCAGCCTGCCCTTG-3'
ScarF2	F: 5'-CTCTTGCGTCTACAGGG-3'
ScarF2	R: 5'-GCTCAGCGGTTTCTATT-3'
Leugpcr	F: 5'-GGTGTTTGTCTGGGTTG-3'
Leugpcr	R: 5'-GGTCTGAGTGAAGAGGGA-3'
ACC	F: 5'-TTAGACCTGGATCAACGGCG-3'
ACC	R: 5'-CATGATCTGTCCTGTACGGG-3'
Heparanase	F: 5'-CAAGCGTTTAGTCACTCGGC-3'
Heparanase	R: 5'-GGTTGCATTCCACGAGTTGTC-3'
Leptin receptor	F: 5'-GTCACACTGATGATGTCACAGAACCAGATG-3'
Leptin receptor	R: 5'-GCTAAAGACCTCTATTACCTCGAGATGACC-3'
C-myc	F: 5'-CCCAGCCGGAGACAGTCGCTCTCCACCGCG-3'
C-myc	R: 5'-CCACAGTCACCACATCAATTTCTTCCTCC-3'

RT-PCR, reverse transcription-polymerase chain reaction; HCV, hepatitis C virus; NS5B, non-structural protein 5B; GFP, green fluorescent protein; F, forward; R, reverse.

Western blot analysis. Zebrafish larvae total protein was extracted using lysis buffer and separated by 12% SDS-PAGE. The protein bands were transferred onto nitrocellulose membranes followed by blocking of the membranes in TBS containing 10% skim milk. The membranes were incubated with anti-core or anti-NS5B antibodies (Abcam) at 1:2,000 dilutions in TBS containing 1% skim milk, and the membranes were then washed and incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgGs (1:2,000 dilutions; Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. Proteins were detected using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) with the AlphaEase® FC Imaging System (Alpha Innotech Corp., San Leandro, CA, USA).

Whole mount in situ hybridization. The core (nt 430-702) and NS5B (nt 8067-8459) gene sequences of the J4L6 strain were used as templates for hybridization probe synthesis, using the

DIG RNA Labeling kit (Roche Diagnostics Scandinavia AB, Bromma, Sweden). Whole mount *in situ* hybridization was performed as previously described (13). Briefly, larvae at 10 dpf were fixed with 4% paraformaldehyde for 10 h at 4˚C and washed with 1X phosphate-buffered saline containing Tween-20 (PBST). The larvae were treated with proteinase K and DNase I separately, pre-hybridized at 65˚C for 4 h, and hybridized with the RNA probes such as *core* and *NS5B* respectively, at 65˚C overnight. The residual probe was washed with 0.2X saline sodium citrate, followed by incubation with anti-Dig-AP (Roche Diagnostics Scandinavia AB) at 4˚C overnight. After washing with 1X PBST, the samples were developed with BCIP/NBT solution for 30 min and this reaction was terminated by washing with 1X PBST. Hybridized signals of the *core* and *NS5B* genes were detected using the purple colorigenic substrate. The larvae were observed under a light microscope (SZ61TRC; Olympus).

Treatment with drugs. Ribavirin and oxymatrine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ribavirin at final concentrations 1000, 100 and 10 μg/ml, and oxymatrine at concentrations of 200, 20 and 2 μg/ml were added to the zebrafish cultivation water, and were incubated with the zebrafish larvae injected or not with pH5B at 5 dpf for another 5 days. The larvae were then collected for analysis.

Results

Replication of the HCV sub-replicon in zebrafish larvae. To ensure that the HCV sub-replicon could be expressed in the larva liver, a liver specific promoter, mHNF4a (14-16), was introduced upstream of the gene expression cassette, followed by the HCV 5'UTR-core, encephalomyocarditis virus (EMCV) IRES, EGFP, EMCV IRES and HCV NS5B 3'UTR genes. The inclusion of the reporter gene encoding EGFP enabled the easy detection of the HCV sub-replicon construct by fluorescence examination (Fig. 1B and C), indicating HCV protein expression. EGFP fluorescence was observed mainly in the liver of the larvae, and to a lesser extent in the intestines and pancreas in the injected zebrafish larvae.

We further examined the transcription of the HCV *core* and *NS5B* genes in zebrafish using the specific primer, NS5B-R, that only binds to the NS5B mRNA 3'end to test the transcript of the sub-replicon, and an universal primer $d(T)_{18}$ by RT-PCR. The results revealed thatthe HCV *core* and *NS5B* genes were expressed in the microinjected larvae, whereas no expression was detected in the wild-type (WT) larva control (Fig. 1D). The HCV sub-replicon transcript contained genetic information for the *5'UTR*, *core*, *EGFP*, *NS5B* and *3'UTR*, which are the elements which respond to HCV RNA replicatiion apart from *EGFP*. When NS5B polymerase is translated, the sub-replicon transcript can be used as a template for the replication of the HCV sub-replicon negative strand that harbors antisense RNA for the core, EGFP and NS5B. Thus, the production of the HCV negative strand RNA is likely to be performed by NS5B since no endogenous RNA-dependent RNA polymerase is known to exist in zebrafish. The negative strand of HCV RNA was detected by reverse transcription *in vitro* using total RNA as a template and the specific forward primer core-F that only binds to the negative strand sequence of the core 3'end to produce the positive strand core cDNA, followed by PCR with the core primer pair and NS5B primer pair to amplify the negative strand sequence. Thus, the products of core, NS5B and EGFP are a sign of the successful replication of the HCV subgenomic replicon in zebrafish (Fig. 1D). No HCV product was detected in the WT zebrafish by RT-PCR (Fig. 1D).

Hybridization *in situ* was carried out for exploring the localization of HCV gene expression in zebrafish. With the antisense RNA probes or the sense RNA probes of the *core* and *NS5B* genes, the hybridized signals were mainly detected in the zebrafish liver, with minor signals detected in the intestines and pancreas of the larvae at 10 dpf (Fig. 1E). By contrast, no HCV signals were detected in the WT generation. By control, the liver-specific gene, *transferrin,* was detected only by the antisense *transferrin* RNA probe, and was shown as negative by the sense *transferrin* RNA probe. These results indicate that the existence of the HCV antisense RNA sequence demonstrates the potential of the HCV sub-replicon to replicate in zebrafish liver (the HCV sense probe results in Fig. 1E).

Furthermore, the HCV core, EGFP and NS5B proteins were examined by western blot analysis of the zebrafish larvae injected with pH5B the WT larva control. Bands of the core, EGFP and NS5B proteins were detected at the predicted sizes with anti-core, anti-EGFP or anti-N5B antibodies, respectively, but no signals were detected in the WT control (Fig. 1F). These result indicates that the HCV sub-replicon was indeed expressed and that its transcript became a RNA template for the successful HCV sub-genome replication in the pH5B-injected zebrafish.

Biological impact of pH5B microinjection into zebrafish larvae. The HCV infection has been reported to cause alterations in the expression of host genes in human liver cells (17). Thus, in this study, we investigated whether the replication of the HCV sub-replicon in the larvae also causes changes in gene expression using RT-PCR. The results are presented in Fig. 2A. The expression of the alpha-2-HS-glycoprotein (*AHSG*) (18), *Hsp70* (19), *chemokine-1* (20), Leucine-rich repeat-containing G protein-coupled receptor 5 (*Leugpcr*) (17), scavenger receptor class F, member 2, (*ScarF2*) (21) and ER lipid raft associated 1 (*Erlin1*) (17) genes were increased in the larvae at 8 and 12 days after the pH5B injection, compared with the untreated WT controls. The results in the zebrafish are in accordance with those from previous studies on HCV-infected human liver cells (17,22), demonstrating the possible biological impact on the host.

To determine the pathological changes in the microinjected zebrafish larvae, we used RT-PCR to detect the expression of the marker genes for liver pathology (8) (Fig. 2B). In the first stage of liver disease, fatty liver, acetyl-CoA carboxylase (ACC) (23) is the limiting enzyme in the biosynthesis of fatty acids, and its high expression can promote the synthesis of fat. Our results revealed that the expression of ACC was almost undetectable in the WT larvae at 8 and 12 dpf, but was significantly increased in the pH5B-injected larvae at 12 dpf compared to the injected larvae at 8 dpf (Fig. 2B). In the second stage of liver disease, liver fibrosis, the leptin receptor (24) and heparanase (25) are known as marker genes and these were selected for examination in our study. Our results revealed that the expression of leptin receptor and heparanase was significantly increased in the

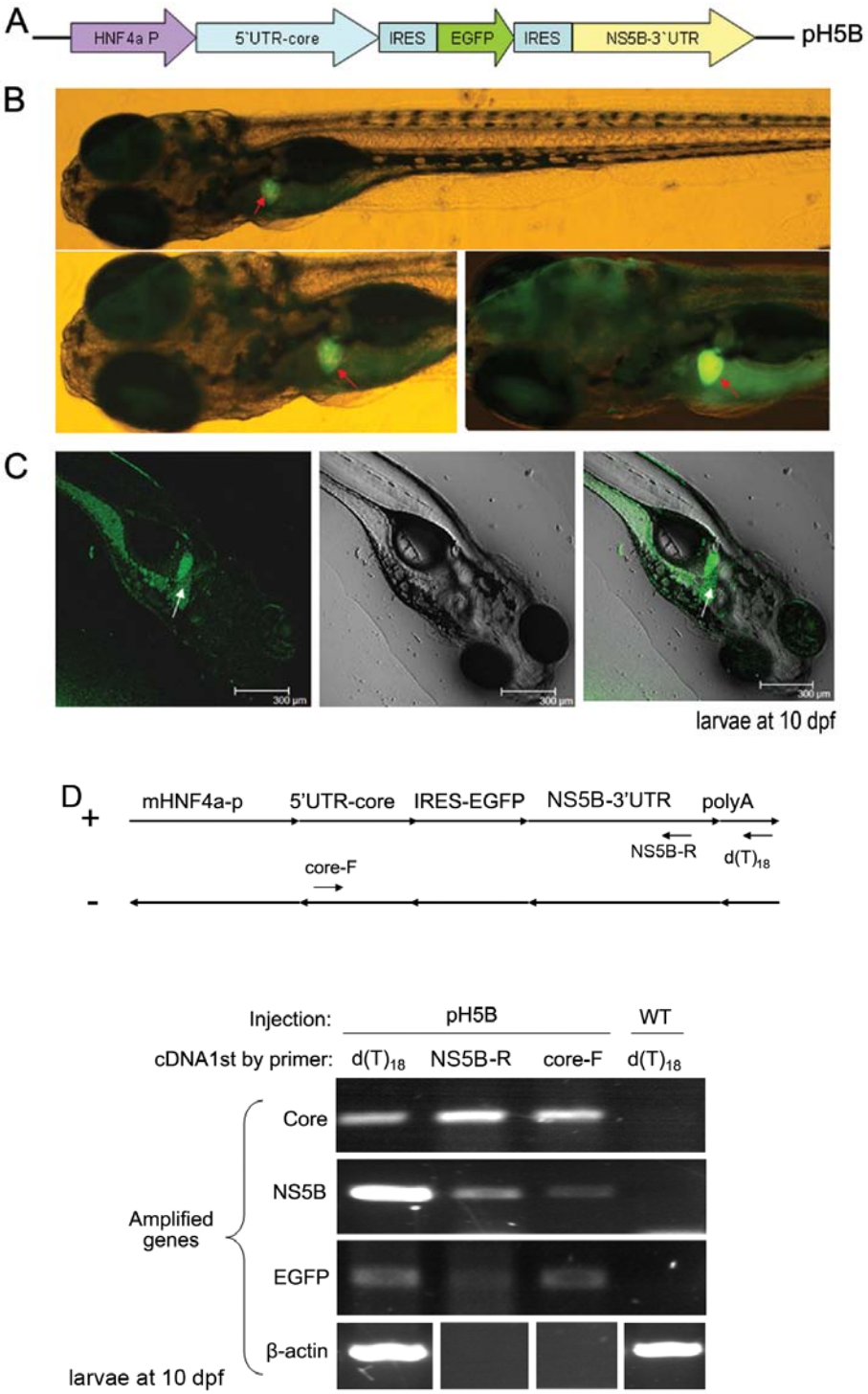

Figure 1. Replication of the hepatitis C virus (HCV) sub-replicon in zebrafish larvae. (A) Schematic diagram of the HCV sub-replicon vector, pH5B. The mouse hepatocyte nuclear factor 4 (mHNF4a) promoter was included for transcription. The HCV 5' untranslated region (5'UTR)-core sequence was inserted downstream of the HNF4a promoter, followed by internal ribosome-entry site (IRES)-enhanced green fluorescent protein (EGFP), and IRES-non-structural protein 5B (NS5B)-3'UTR. (B) Fluorescence microscopic observation of the HCV sub-replicon in the liver of pH5B-injected zebrafish larvae at 10 days post-fertilization (dpf) using a green fluorescent protein (GFP) filter (480 nm excitation, 505 nm emission; image, x100, original). Red arrows indicate positive signals of GFP in the liver. (C) Confocal DIC images of pH5B-injected zebrafish larvae at 10 dpf. The left, the middle and the right images are at fluorescence field, bright field and the overlay, respectively. White arrows indicate the positive signal of GFP in the liver, but are different parts from the fluorescent images of the liver shown in (B). (D) Upper panel shows templates and primers for the reverse transcription reactions; lower panel shows reverse transcription-polymerase chain reaction (RT-PCR) results of the target genes, *core*, *NS5B* and *EGFP*, as well as β-actin at 10 dpf in the zebrafish larvae injected with the HCV sub-replicon vector. β-actin was used as a loading control.

pH5B-injected larvae compared with the WT larvae (Fig. 2B). In the third stage of liver disease, hepatocellular carcinoma, the level of c-myc and survivin (26) in the injected larvae was similar to that in the WT larvae at 12 dpf. These findings indicate that the zebrafish HCV sub-replicon model can partly mimic HCV activity in the human liver.

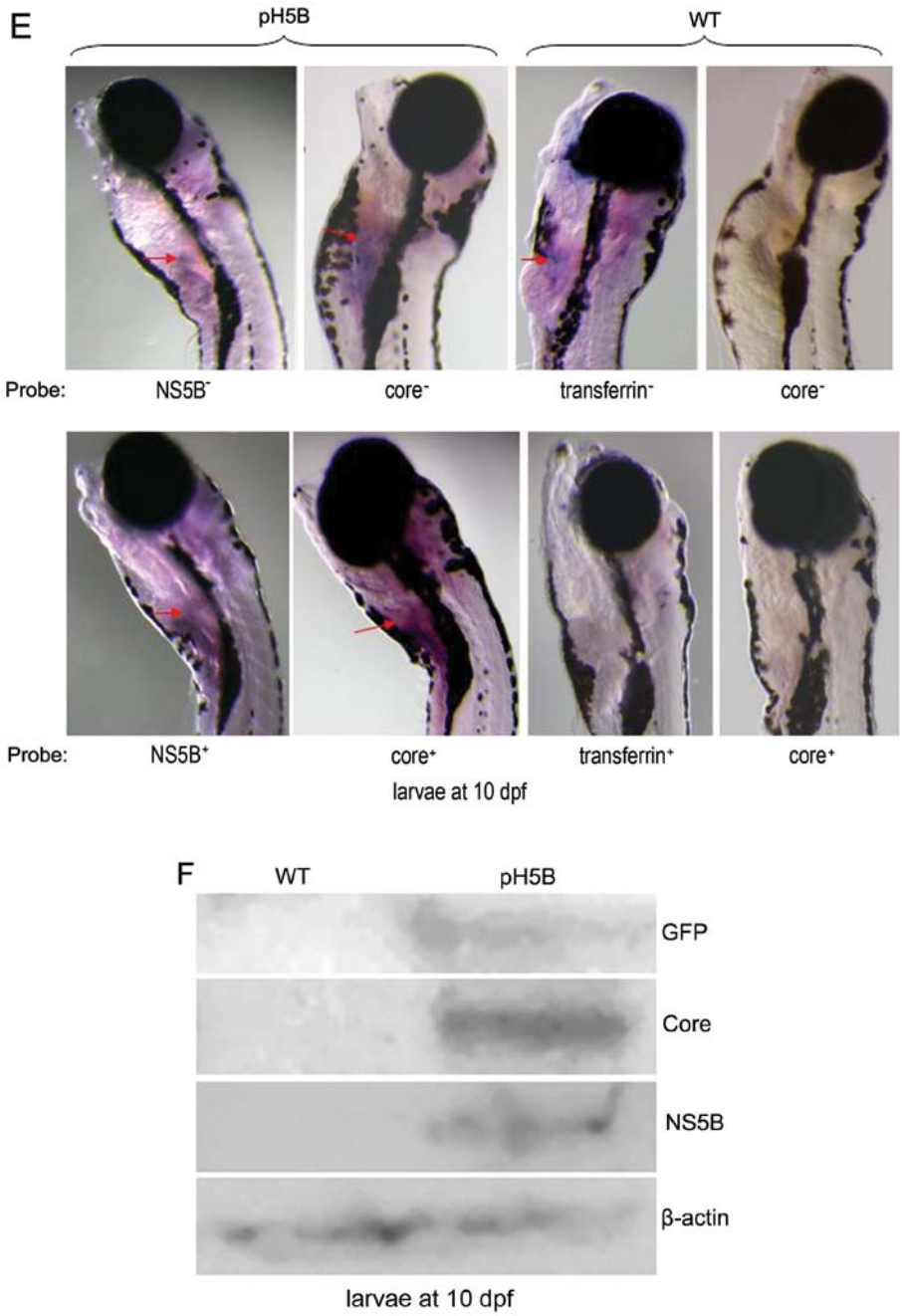

Figure 1. Continued. (E) Whole mount *in situ* hybridizations were performed on larvae at 10 dpf using antisense or sense RNA probes. Red indicate show the positive signals (including NS5B-positive or -negative, core-positive or -negative, and transferrin-positive) in the larvae livers (x80 magnification, original). (F) Western blots of the GFP, HCV core and NS5B proteins from the larvae at 10 dpf were detected using anti-EGFP, anti-core and anti-NS5B antibodies, respectively. β-actin was used as a loading control.

Evaluation of the zebrafish HCV sub-replication model with anti-HCV drugs. The larvae displaying EGFP fluorescence were treated with various concentrations of ribavirin or oxymatrine from 5 to 10 dpf, and the core gene was then tested as the sign of the evaluation of the HCV sub-replicon model by RT-PCR. Incubation of the larvae in the ribavirin- or oxymatrine-containing water for 5 days markedly inhibited the amplification of the negative strand core RNA of the HCV sub-replicon (Fig. 3). The inhibition occurred in a dose-dependent manner both in the ribavirin- and oxymatrine-treated groups. Ribavirin at 100 μg/ml, or oxymatrine at concentrations ≥20 μg/ml, significantly suppressed the replication of the HCV sub-replicon. The positive strand core RNA is included in the figure as a reference to show the successful microinjection and transcription of the pH5B vector into the zebrafish larvae (Fig. 3). As the positive strand HCV core RNA maintained a steady level during the course of treatment, the anti-HCV mechanisms of the 2 drugs apperared to be associated with HCV sub-genomic replication-related events. None of the larvae died or grew abnormally during the course of treatment, indicating a good level of safety of the drugs at the doses used. These results indicate that the zebrafish-hosted HCV sub-replicon amplification system is a suitable animal model with which to evaluate anti-HCV drugs or drug candidates, particularly in the case of water-soluble agents.

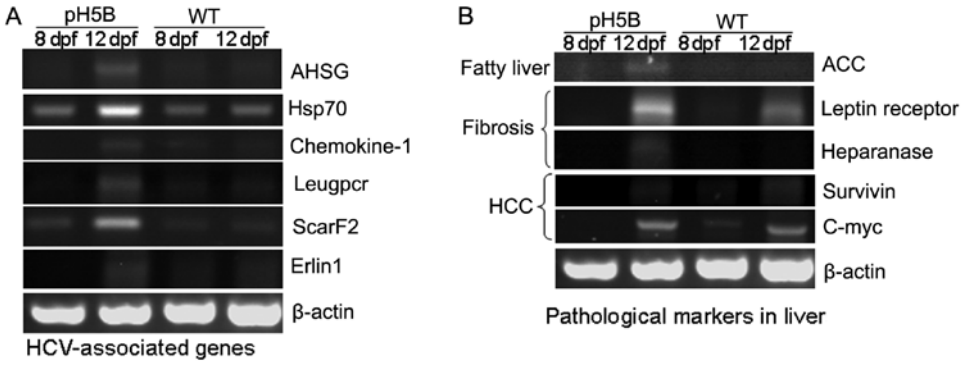

Figure 2. Biological response of zebrafish larvae injected with the hepatitis C virus (HCV) sub-replicon vector. (A) Expression of the HCV-associated host genes in zebrafish larvae. The transcription levels of the genes selected were examined by reverse transcription-polymerase chain reaction (RT-PCR) in the wild-type (WT) and pH5B-injected larvae (at 8 and 12 dpf). (B) Expression of the liver pathological marker genes in pH5B-injected larvae (at 8 and 12 dpf) was compared with that in WT larvae by RT-PCR. dpf, days post-fertilization.

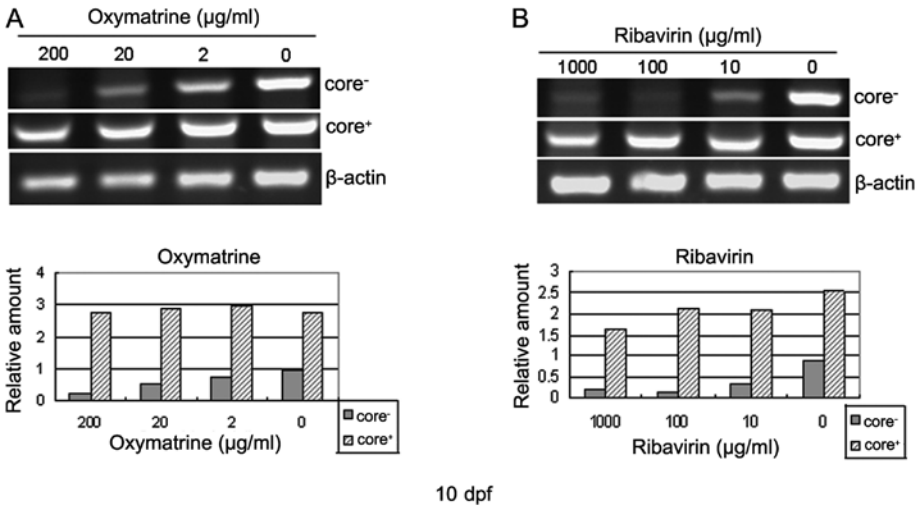

Figure 3. Inhibitory effects of ribavirin and oxymatrine on the amplification of the hepatitis C virus (HCV) sub-replicon in zebrafish larvae. Zebrafish larvae injected with pH5B were treated with ribavirin or oxymatrine from 5 to 10 dpf, and HCV core RNA was measured by reverse transcription-polymerase chain reaction (RT-PCR) to investigate the anti-HCV effect of the drugs. $Core^+$, the positive strand of the core RNA; $core^-$, the negative strand of the core RNA. (A) Effects of oxymatrine and (B) effects of ribavirin. Both histograms indicate the effects of the drugs on the HCV replication level [dose-dependent decrease ($core^-$)]; the transcription levels of the core positive strand ($core^+$) remained relatively stable; both were normalized to β-actin. dpf, days post-fertilization.

Discussion

In this study, we developed a zebrafish HCV subgenomic replicon model that can be transcribed by the mHNF4a promoter. All the proteins encoded by the HCV sub-replicon construct were detected in the microinjected zebrafish larvae by western blot analysis, which indicates that the mHNF4a promoter functioned properly in zebrafish liver and that the key factors for HCV RNA repliction, core and HCV RNA-dependent polymerase (NS5B) were successfully translated. As the negative strand RNA was an intermediate product of replication, its existence demonstrates that the HCV sub-genome can be replicated in zebrafish. These results indicated the successful development of a zebrafish HCV subgenomic replication model. Moreover, the changes observed in the transcription levels of HCV-associated genes and the liver pathological marker genes in the pH5B-injected zebrafish larvae are in agreement with those observed in HCV-infected human liver cells (17), which further confirm the successful creation of the zebrafish model of HCV.

The inhibitory effects of ribavirin and oxymatrine, two anti-virus drugs used in clinical practice, against the HCV sub-genomic replication indicate that the zebrafish HCV sub-replicon model may serve as a valuable platform with which to study the molecular events in HCV genomic replication and to evaluate preventive and therapeutic strategies to combat HCV infection. Furthermore, the results of this study confirm the suitability of zebrafish as a HCV small animal model. Compared with mouse models of HCV (27-30), this model system has several advantages for drug screening and evaluation. First, the HCV sub-replicon replicates actively and steadily in zebrafish tissue; second, the procedure for creating the sub-replicon-positive larva is straight forward and simple; and third, this easy-to-handle small biological model seems to be suitable for drug screening. The major disadvantages of this model are that the HCV sub-replicon is possibly more suitable for water-soluble compounds and the dose calculation is complicated.

Our results also provide evidence that zebrafish liver cells may contain biological circumstances compatible to human

HCV replication that usually occur in human hepatocytes. Although there may be other viral or host factors that contribute to HCV RNA replication, the HCV core and NS5B proteins together with the HCV 5'UTR and 3'UTR RNA sequence are capable of copying the subgenomic RNA in the zebrafish model, and similar results were reported in the study by Lee *et al* (6).

In conclusion, in this study, we confirm the use of zebrafish as an *in vivo* biological model system for HCV replication, with potential applications in the evaluation of anti-HCV drugs.

Acknowledgements

This study was supported by the 'Innovative Group Grant' from the Ministry of Education (China), the 11th 5-year 'New Drug R&D Program' of the Ministry of Science and Technology (China) (J.-D.J.), a National S&T Major Special Project on Major New Drug Innovation grant (China) (item no. 2009ZX09301-003-6-2) (J.-P.Z.) and the National Natural Science Foundation of China (no. 30772681) (J.-P.Z.). We would also like to thank Wei-Xian Wang and Jie Meng for their assistance with the microinjection of the zebrafish.

References

1. Gottwein JM and Bukh J: Hepatitis C virus host cell interactions uncovered. Proc Natl Acad Sci USA 104: 13215-13216, 2007.
2. Friebe P, Lohmann V, Krieger N and Bartenschlager R: Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. J Virol 75: 12047-12057, 2001.
3. Lee KJ, Choi J, Ou JH and Lai MM: The C-terminal transmembrane domain of hepatitis C virus (HCV) RNA polymerase is essential for HCV replication in vivo. J Virol 78: 3797-3802, 2004.
4. Behrens SE, Tomei L and De Francesco R: Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J 15: 12-22, 1996.
5. Lohmann V, Körner F, Herian U and Bartenschlager R: Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol 71: 8416-8428, 1997.
6. Lee S, Lee JH, Kee YH, Park MY and Myung H: Partial reconstitution of hepatitis C virus RNA polymerization by heterologous expression of NS5B polymerase and template RNA in bacterial cell. Virus Res 114: 158-163, 2005.
7. Liang JO and Rubinstein AL: Patterning of the zebrafish embryo by nodal signals. Curr Top Dev Biol 55: 143-171, 2003.
8. Rekha RD, Amali AA, Her GM, *et al*: Thioacetamide accelerates steatohepatitis, cirrhosis and HCC by expressing HCV core protein in transgenic zebrafish *Danio rerio*. Toxicology 243: 11-22, 2008.
9. Wallace KN and Pack M: Unique and conserved aspects of gut development in zebrafish. Dev Biol 255: 12-29, 2003.
10. Amatruda JF, Shepard JL, Stern HM and Zon LI: Zebrafish as a cancer model system. Cancer Cell 1: 229-231, 2002.
11. Lam SH, Wu YL, Vega VB, *et al*: Conservation of gene expression signatures between zebrafish and human liver tumors and tumor progression. Nat Biotechnol 24: 73-75, 2006.
12. Ding CB, Zhang JP, Zhao Y, Peng ZG, Song DQ and Jiang JD: Zebrafish as a potential model organism for drug test against hepatitis C virus. PLoS One 6: e22921, 2011.
13. Thisse C and Thisse B: High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc 3: 59-69, 2008.
14. Quasdorff M, Hösel M, Odenthal M, *et al*: A concerted action of HNF4alpha and HNF1alpha links hepatitis B virus replication to hepatocyte differentiation. Cell Microbiol 10: 1478-1490, 2008.
15. Qadri I, Iwahashi M, Kullak-Ublick GA and Simon FR: Hepatocyte nuclear factor (HNF) 1 and HNF4 mediate hepatic multidrug resistance protein 2 up-regulation during hepatitis C virus gene expression. Mol Pharmacol 70: 627-636, 2006.
16. Bailly A, Elena M, Torres-Padilla, Tinel AP and Weiss MC: An enhancer element 6 kb upstream of the mouse HNF4α1 promoter is activated by glucocorticoids and liver-enriched transcription factors. Nucleic Acids Res 29: 3495-3505, 2001.
17. Nishimura-Sakurai Y, Sakamoto N, Mogushi K, *et al*: Comparison of HCV-associated gene expression and cell signaling pathways in cells with or without HCV replicon and in replicon-cured cells. J Gastroenterol 45: 523-536, 2010.
18. Yilmaz Y, Yonal O, Kurt R, *et al*: Serum fetuin A/α2HS-glycoprotein levels in patients with non-alcoholic fatty liver disease: relation with liver fibrosis. Ann Clin Biochem 47: 549-553, 2010.
19. Parent R, Qu X, Petit MA and Beretta L: The heat shock cognate protein 70 is associated with hepatitis C virus particles and modulates virus infectivity. Hepatology 49: 1798-1809, 2009.
20. Helbig KJ, Ruszkiewicz A, Semendric L, Harley HA, McColl SR and Beard MR: Expression of the CXCR3 ligand I-TAC by hepatocytes in chronic hepatitis C and its correlation with hepatic inflammation. Hepatology 39: 1220-1229, 2004.
21. Chang ML, Yeh CT, Chen JC, *et al*: Altered expression patterns of lipid metabolism genes in an animal model of HCV core-related, nonobese, modest hepatic steatosis. BMC Genomics 9: 109, 2008.
22. Blais DR, Brûlotte M, Qian Y, Bélanger S, Yao SQ and Pezacki JP: Activity-based proteome profiling of hepatoma cells during hepatitis C virus replication using protease substrate probes. J Proteome Res 9: 912-923, 2010.
23. Shieh YS, Chang YS, Hong JR, *et al*: Increase of hepatic fat accumulation by liver specific expression of hepatitis B virus X protein in zebrafish. Biochim Biophys Acta 1801: 721-730, 2010.
24. Cheng SP, Chi CW, Tzen CY, *et al*: Clinicopathologic significance of leptin and leptin receptor expressions in papillary thyroid carcinoma. Surgery 147: 847-853, 2010.
25. Tsiperson V, Goldshmidt O, Ilan N, *et al*: Heparanase enhances early hepatocyte inclusion in the recipient liver after transplantation in partially hepatectomized rats. Tissue Eng Part A 14: 449-458, 2008.
26. Ma AC, Chung MI, Liang R and Leung AY: The role of survivin2 in primitive hematopoiesis during zebrafish development. Leukemia 23: 712-720, 2009.
27. Ploss A and Rice CM: Towards a small animal model for hepatitis C. EMBO Rep 10: 1220-1227, 2009.
28. Park IW, Ndjomou J, Fan Y, Henao-Mejia J and He JJ: Hepatitis C virus is restricted at both entry and replication in mouse hepatocytes. Biochem Biophys Res Commun 387: 489-493, 2009.
29. Lerat H, Kammoun HL, Hainault I, *et al*: Hepatitis C virus proteins induce lipogenesis and defective triglyceride secretion in transgenic mice. J Biol Chem 284: 33466-33474, 2009.
30. Guévin C, Lamarre A and Labonté P: Novel HCV replication mouse model using human hepatocellular carcinoma xenografts. Antiviral Res 84: 14-22, 2009.