Chemical inhibition reveals differential requirements of signaling pathways in \( \text{kras}^{\text{V12}} \)- and \( \text{Myc} \)-induced liver tumors in transgenic zebrafish

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Previously we have generated inducible liver tumor models by transgenic expression of an oncogene and robust tumorigenesis can be rapidly induced by activation of the oncogene in both juvenile and adult fish. In the present study, we aimed at chemical intervention of tumorigenesis for understanding molecular pathways of tumorigenesis and for potential development of a chemical screening tool for anti-cancer drug discovery. Thus, we evaluated the roles of several major signaling pathways in \( \text{kras}^{\text{V12}} \)- or \( \text{Myc} \)-induced liver tumors by using several small molecule inhibitors: SU5402 and SU6668 for VEGF/FGF signaling; IWR1 and cardionogen 1 for Wnt signaling; and cyclopamine and Gant61 for Hedgehog signaling. Inhibition of VEGF/FGF signaling was found to deter both \( \text{Myc} \)- and \( \text{kras}^{\text{V12}} \)-induced liver tumorigenesis while suppression of Wnt signaling relaxed only \( \text{Myc} \)- but not \( \text{kras}^{\text{V12}} \)-induced liver tumorigenesis. Inhibiting Hedgehog signaling did not suppress either \( \text{kras}^{\text{V12}} \) or \( \text{Myc} \)-induced tumors. The suppression of liver tumorigenesis was accompanied with a decrease of cell proliferation, increase of apoptosis, distorted liver histology. Collectively, our observations suggested the requirement of VEGF/FGF signaling but not the hedgehog signaling in liver tumorigenesis in both transgenic fry. However, Wnt signaling appeared to be required for liver tumorigenesis only in \( \text{Myc} \) but not \( \text{kras}^{\text{V12}} \) transgenic zebrafish.

Hepatocellular carcinoma (HCC), a major liver malignance, is a global health problem\(^1\)–\(^4\). With the advancement in anti-cancer therapies in the past two decades, mortality from most malignancies declined steadily\(^5\); however, HCC-related death increased significantly from 1990 to 2015 in some parts of the world such as United States\(^5,6\). Poor prognosis is primarily due to limited understanding of the disease. HCC is highly heterogeneous in both pathology and molecular pathways due to patient genetic backgrounds and multiple risk factors; as a result, HCC is resistant to both standard chemotherapy and radiotherapy\(^7\). Nowadays, surgical resection and liver transplantation remain the best treatment options\(^8\).

In recent years, increasing research efforts have been made for understanding of the underlying molecular mechanisms causing the initiation and progression of HCC. It has been found that growth factor, MAPK, PI3K, mTOR and WNT pathways are among the most important\(^8\)–\(^11\). However, translational medicine developed from molecular understandings is still limited. Till date, only a single targeted therapy drug, sorafenib, a multikinase inhibitor, has been approved by US Food and Drug Administration (FDA) as a targeted therapeutic drug for HCC. Thus, more research is required to understand the underlying molecular aberrations of HCC, specifically under different oncogenes, for new drug discovery.

In the past few years, we have generated several inducible liver tumor models by transgenic expression of a selected oncogene in hepatocytes in zebrafish\(^12\)–\(^16\). In these tumor models, rapid hepatocarcinogenesis is observed, with full-blown carcinoma in a few weeks upon activation of an oncogene. In addition, with the inducible system, the activation of an oncogene can be temporally controlled, thus providing an excellent platform to study
cancer initiation events. In this study, two oncogene transgenic lines, Tg(fabp10:rtTA2s-M2; TRE2:EGFP-krasV12) (gz32Tg) and Tg(fabp10:TA; TRE:myc; CK:RFP) (gz26Tg) in a Tet-On system to control the hepatocyte-specific expression of oncogenic krasV12 or Myc respectively12,14, were employed and they are termed as kras+ and Myc+, respectively in this report. krasV12- or Myc-induced HCC have been found as an elevated MAPK/ERK and MYC signaling in approximately 30% and 70% of HCC patients respectively17,18. Transcriptional analyses of our transgenic zebrafish models indicated that krasV12- and Myc-induced zebrafish HCC shared conserved gene expression signatures with 23.5% and 23.8% of human HCC, respectively19. In addition, one reporter transgenic line, Tg(fabp10:DsRed; elA:GFP) (gz15Tg) with DsRed-labeled liver and GFP-labeled exocrine pancreas20, was used as a normal control for the liver morphology and referred as fabp10+.

Here we demonstrated the feasibility of using small chemical inhibitors to suppress oncogenic growth of livers in our previously created zebrafish liver tumor models driven by krasV12 and Myc oncogenes12,14. These chemical inhibitors targeted three popular molecular pathways in carcinogenesis, VEGF/FGF, Wnt and Hedgehog. We observed differential requirements of these molecular pathways in the two tumor models. While VEGF/FGF was required for both krasV12- and Myc-driven tumors, Hedgehog signaling appeared to be dispensable in both types of tumors. In contrast, WNT signaling was required for Myc-induced but not for krasV12-induced tumors. Our studies indicate the possible development of chemical screening platform using these oncogene transgenic zebrafish models for rapid and high-throughput anti-cancer drug discovery.

Results
Inhibition of VEGF/FGF pathway suppresses both krasV12- and Myc-induced oncogenic liver enlargement. To investigate the role of VEGF/FGF pathways in our liver tumors models, two chemical inhibitors, SU6668 and SU5402, were used. SU6668 is a VEGF pathway inhibitor but also has binding activity to FGF receptor21. Similarly, SU5402 has been shown to potently inhibit FGF signaling and is also known to cross-react with VEGF receptor22. 1 μM SU5402 or 1 μM SU6668 was used together with doxycycline (Dox) to treat kras+ and Myc+ larvae from 4 dpf to 7 dpf. In fabp10+ control larvae, liver morphology in lateral view, as denoted by RFP expression at 7 dpf, displayed a hooked shape even in the presence of Dox (Fig. 1A). Expression of either krasV12 or Myc oncogene resulted in an obvious and significant enlargement of the liver with a round, ball-like appearance (Fig. 1D,G). In fabp10+ control larvae, co-treatment with SU5402/Dox or SU6668/Dox did not cause an obvious change of liver morphology (Fig. 1B,C). In contrast, in both kras+ and Myc+ larvae co-treated with either SU5420/Dox or SU6668/Dox, normal liver outline was largely restored (Fig. 1E,F,H,I), bearing close resemblance to the wild type larvae. 2-D measurement of liver sizes based on the GFP or RFP expression confirmed that the exposure to Dox significantly increased liver size in both kras+ and Myc+ larvae while co-treatments with either inhibitor significantly reduced the liver enlargement caused by oncogene induction in both kras+ and Myc+ larvae (Fig. 1J,K). These observations suggested that the inhibition of VEGF/FGF pathway in both krasV12-, or Myc-induced tumorigenesis was capable of abrogating the oncogene-induced liver enlargement.

Inhibition of Wnt pathway suppresses Myc- but not kras-induced oncogenic liver enlargement. Aberrant Wnt signaling as a consequence of either KRAS or MYC oncogene activation or as an inducer of Myc expression has been previously reported in human HCC12,24. To test if the Wnt pathway played a role in krasV12- or Myc-induced carcinogenesis, two potent inhibitors of the Wnt pathway, IWR1 and cardionogen 1, were used to treat both kras+ and Myc+ larvae. IWR1 abrogates Axin protein turnover and stabilizes the Axin destruction complex, thus promoting β-catenin degradation25 while cardionogen 1 has been postulated to decrease TCF/Lef activity and thus to reduce effect of β-catenin initiated gene transcription25,26. Neither of the inhibitors showed significant effect on liver morphology in fabp10+ control larvae (Fig. 2A–C). However, in oncogenic larvae, the two inhibitors showed different effects on kras+ and Myc+ larvae. As shown in Fig. 2E,F, neither IWR1 nor Cardionogen 1 treatment could deter krasV12-induced enlargement of liver. Morphologically, these kras+ larvae exposed to IWR1/Dox or Cardionogen 1/Dox retained enlarged livers (Fig. 2E,F), similar to the Dox alone controls (Fig. 2D). In contrast, both inhibitors significantly suppressed liver enlargement in Myc+ larvae (Fig. G–I). 2D liver size measurement confirmed that there was no significant reduction in liver size by the two inhibitors in the kras+ larvae (Fig. 2J). However, there was indeed significant reduction of liver size by the two inhibitors in the Myc+ larvae (Fig. 2K). Thus, Wnt signaling pathway was essential for Myc-induced but not for krasV12-induced liver enlargement at least at the initial stage of liver tumorigenesis.

Inhibition of hedgehog pathway fails to suppress both krasV12- and Myc-induced liver enlargements. Activating mutations of the hedgehog pathway have long been identified as an important cause for carcinogenesis in a variety of cancers27. To elucidate the role of the hedgehog pathway in krasV12- and Myc-induced carcinogenesis, two inhibitors of the Hedgehog pathway, cyclopamine (a Smoothened protein inhibitor) and GANT61 (a Gli protein inhibitor) were used to treat kras+ and Myc+ larvae. As shown in Fig. 3A–C, the two inhibitors did not show any significant effect on liver morphology in fabp10+ control larvae. In oncogenic larvae, neither of the inhibitors was able to suppress the oncogene-induced liver enlargement in kras+ or Myc+ larvae (Fig. 3D–I). Cyclopamine/Dox or GANT61/Dox treated kras+ or Myc+ larvae retained the enlarged round liver morphology that was typically observed in oncogenic liver at this stage. 2D liver size measurement further confirmed that the liver sizes of cyclopamine/Dox and GANT61/Dox treated kras+ and Myc+ larvae were indifferent from those of the kras+ or Myc+ larvae treated with Dox alone; thus, inhibition of Hedgehog pathway did not suppress the oncogene induced liver enlargement (Fig. 3J,K).

The alteration of liver size is mainly contributed by change of cell proliferation. Aberrant cell cycle control is a major hallmark of carcinogenesis1. To investigate if the gross liver enlargements observed in kras+ and Myc+ larvae were a consequence of aberrant cell cycle in the liver, PCNA staining for proliferative cells

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and TUNEL assay for apoptotic cells were carried out. As shown in Fig. 4A,E,I, both kras+ and Myc+ larvae after Dox induction showed a significant increase in proliferating cells as compared to wild type (WT) controls. By quantification, induced kras+ and Myc+ larvae had increases of proliferating cells by about 10 fold (Fig. 4M,N). Exposure to each of the three signaling pathway inhibitors (SU5402, IWR1 or cyclopamine) in WT control larvae did not alter the number of proliferating cells (Fig. 4B–D). When kras+ and Myc+ larvae were exposed to SU5402, the numbers of proliferating cells in the liver were greatly reduced compared to that in the Dox-induced tumor controls (Fig. 4F,J). In the presence of IWR1, the number of proliferating cells was reduced in the Myc+ larvae but not in the kras+ larvae (Fig. 4G,K), while in the presence of cyclopamine, the number of proliferating cells showed no decrease in both kras+ and Myc+ larvae (Fig. 4H,L). All of these observed trends were further confirmed by quantification of the number of proliferating cells based on per square micrometers (Fig. 4M,N). Overall, these data were consistent with the observations of liver sizes in the presence of these three types of inhibitors as shown in Figs 1, 2 and 3; therefore, the inhibition of liver enlargement was achieved by inhibition of cell proliferation.

As shown in Fig. 5, apoptosis of liver cells was also examined by TUNEL assay for the same set of samples analyzed in Fig. 4. In general, there were a low number of apoptotic cells in non-oncogenic livers in WT control larvae treated with Dox (Fig. 5A). Induction of oncogene expression in both kras+ and Myc+ larvae also induced

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**Figure 1. Effect of inhibition of VEGF/FGF on krasV12- and Myc-induced liver enlargement.** 7 dpf fabp10+, kras+ or Myc+ larvae were treated with either 1 μM SU5402 or 1 μM SU6668 in the presence of 10 μg/ml Dox and 2D liver size was measured based on images. (A–C) Representative images of 7 dpf fabp10+ control larvae. (D–F) Representative images of 7 dpf kras+ larvae. (G–I) Representative images of 7 dpf Myc+ larvae. (J) Quantification of liver sizes for kras+ larvae (K) Quantification of liver sizes for Myc+ larvae. N = 20 from each groups; statistical significance: *p < 0.05, Scale bar = 20 μm.
an obvious increase of apoptotic cells (Fig. 5E,I). This is consistent with our earlier observation in another oncogene transgenic line, xmrk-induced HCC. Both kras and Myc oncogenes have been reported to be able to induce apoptosis via Rassf1/Nore1/Mst1 and p53 pathways respectively. None of the three inhibitors, SU5402, IWR1 and cyclopamine, affected the numbers of apoptotic cells in WT control larvae, but they did show variable effects on the numbers of apoptotic cells in Dox-treated kras+ and Myc+ larvae. In Dox-induced kras+ larvae, both SU5402 and IWR1 showed mild, but significant, reduction of apoptotic cells in the oncogenic liver (Fig. 5E,F); however, cyclopamine did not reduce the numbers of apoptotic cells (Fig. 5H,M). In Dox-induced Myc+ larvae, SU5402 and IWR1 treatments similarly and more profoundly reduced the number of apoptotic cells (Fig. 5J,K,N), but again cyclopamine had no significant effect on the number of apoptotic cells (Fig. 5L,N). Overall, the state of apoptosis in kras+ and Myc+ larvae were not always consistent with the overall changes of liver size in corresponding groups, but it is interesting to note that in general, the numbers of apoptotic cells in the livers were 10 fold lower than the number of proliferating cells; thus, the changes of liver size was mainly contributed by cell proliferation.

Figure 2. Effect of inhibition of Wnt signaling pathway on krasV12- and Myc-induced liver enlargement. 7 dpf fabp10+, kras+ or Myc+ larvae were treated with either 10μM IWR1 or 10μM Cardionogen 1 in the presence of 10μg/ml Dox and 2D liver size was measured based on images. (A–C) Representative images of 7 dpf fabp10+ control larvae. (D–F) Representative images of 7 dpf kras+ larvae. (G–I) Representative images of 7 dpf Myc+ larvae. (J) Quantification of liver sizes for kras+ larvae. (K) Quantification of liver sizes for Myc+ larvae. N = 20 from each groups; statistical significance: *p < 0.05, Scale bar = 20μm.
Partial reversal of histological features of hyperplasic livers by chemical inhibitors. In order to examine if the suppression of \textit{kras}^{V12} and \textit{Myc}-induced liver enlargement by different small molecule inhibitors correspond to a corresponding changes of altered histopathology, H&E staining of these larvae was carried out. In 7 dpf WT control larvae, a normal liver histology was observed. Hepatocytes were regularly organized as two-cell plates with eosinophilic cytoplasm and round nuclei (Fig. 6A). After either \textit{kras}^{V12} or \textit{Myc} induction, liver histology was changed dramatically. As shown in Fig. 6E,I, both oncogene-induced hepatocytes were less eosinophilic with distorted hepatocyte plates and variable sizes of nuclei. Their nuclei contained visible nucleoli (Fig. 6A–C), implying active transcription and mRNA synthesis. Increased vacuolation was also observed in the liver, suggesting the possibility of abnormal lipid or glycogen accumulation. These histopathological features were largely consistent with human HCC. The dense and irregular nuclei were marks of hyperplasia for active cell proliferation (Fig. 6E,I). In Dox induced \textit{kras}+ and \textit{Myc}+ larvae, all larvae examined had hyperplastic liver histology (Fig. 6M,N).

Treatments with SU5402, IWR1 or cyclopamine showed that none of them could alter the liver histology in WT control larvae (Fig. 6B–D). However, in \textit{kras}+ larvae treated with SU5402, 20% of the larvae reverted to a...
normal histology resembling that of the WT sibling treated with Dox (Fig. 6F,M), with the remaining 80% of the larvae showing liver hyperplasia. In kras+ larvae exposed to IWR1 or Cyclopamine, all of these larvae displayed hyperplastic liver histology (Fig. 6G,H,M). In SU5402 or IWR1 exposed Myc+ larvae, 30% or 10% of the larvae showed a reversion to normal liver histology with the remaining 70% or 90% of the larvae still at liver hyperplasia (Fig. 6J,K,N). Cyclopamine treatment failed to relax the histology of any Myc+ larvae (Fig. 6L,N). 100% of the larvae displayed abnormal histopathology similar to that observed in the Dox induced Myc+ control (Fig. 6L,N).

In general, histological analysis showed that the inhibitors that could deter kras+ or Myc-induced liver enlargement could also relax the oncogene induced histopathological changes to a certain extent.

Figure 4. Cell proliferation analysis of krasV12- and Myc-induced carcinogenesis. 7 dpf wild type (WT), kras+ or Myc+ larvae were treated with 10μM SU5402, 10μM IWR1 or 10μM cyclopamine in the presence of 10μg/ml Dox. Cell proliferation was analyzed by immunohistochemical staining with PCNA primary antibody. (A–D) Representative liver image of 7 dpf WT larvae. (E–H) Representative liver image of 7 dpf kras+ larvae. (I–L) Representative liver image of 7 dpf Myc+ larvae. (M) Statistical analysis of numbers of proliferating cells in the livers of kras+ larvae. (N) Statistical analysis of numbers of proliferating cells in the livers of Myc+ larvae. N = 20 from each groups; statistical significance: *p < 0.05, Scale bar = 20μm.
Discussion
In this study, by using kras+ and Myc+ larvae, visible and significant liver enlargement caused by overexpression of an oncogene can be conveniently and rapidly observed within 4 days of induction in live larvae. Our studies also demonstrated the correlation between liver sizes and severity of liver hyperplasia. Interestingly, some small molecules that are known to suppress a specific molecular pathway could effectively reduce liver size, which was primarily due to the reduction of cell proliferation; as a result, normal liver histology was also partially restored. Inhibition of FGF/VEGF signaling relaxed both krasV12- and Myc- induced hepatocarcinogenesis
while suppression of Wnt signaling only alleviated Myc-induced, but not krasV12-induced, hepatocarcinogenesis, suggesting the specificities of these chemical inhibitors and their specific effects on molecular pathways. Both kras and Myc oncogenes have been reported to regulate VEGF production by activation of MEK, which in turn promote carcinogenesis. Our observation that VEGF/FGF plays a crucial role for both kras- and Myc-initiated hepatocarcinogenesis was consistent with these reports. In contrast, cooperation between the Wnt pathway and Myc is required for cellular transformation and increases cancer frequency in mice. Myc but not Kras has also been reported to interact closely with Wnt pathway while the Wnt pathway enhances Myc expression via a β-catenin mediated mechanism. Moreover, Kras has been reported to promote tumorigenicity by suppression of Wnt signaling. Thus, our observation that Wnt signaling is important for Myc- but not kras-induced tumorigenesis was also consistent with these previously reported studies. In contrast, although Kras or Myc had been reported to activate hedgehog signaling in malignancies such as pancreatic cancer or lymphoma, it appears that Hedgehog signaling is disposable in kras or Myc-induced HCC.

Figure 6. Histological examination of krasV12- and Myc-induced carcinogenesis. 7 dpf WT, kras+ and Myc+ larvae were treated with 10μM SU5402, 10μM IWR1 or 10μM cyclopamine in the presence of 10μg/ml Dox, and subjected histological analysis. (A–D) Representative liver images of 7 dpf WT larvae. Inset in (A) is a magnified area in the box with arrows pointing nucleoli. (E–H) Representative liver images of 7 dpf kras+ larvae. Inset in (E) is a magnified area in the box with arrows pointing to nucleoli of condensed nuclei. (I–L) Representative liver images of 7 dpf Myc+ liver larvae. Inset in (I) is a magnified area in the box with arrows pointing to nucleoli of condensed nuclei. (M) Quantification of liver histology observed for kras+ larvae. (N) Quantification of liver histology observed for Myc+ larvae. N = 10 from each group; scale bar = 20μm.
Previously, we have demonstrated that both \( kras^{V12} \) and \( Myc \) oncogenes are capable of inducing tumorigenesis by overexpression in both juvenile and adult transgenic zebrafish\(^{12,14} \). One advantage of our oncogene transgenic model is the inducibility of oncogene expression and thus the temporal control of tumorigenesis. Now we demonstrated the feasibility for induction of onset of tumorigenesis and chemical intervention in the larva stage. Thus, these transgenic zebrafish should provide convenient \( in \) \( vivo \) tumor models for dissection of molecular pathways involved in tumorigenesis, complementary to popularly used \( in \) \( vitro \) cancer cell models. In particular, the zebrafish has been widely hailed as a potentially high-throughput model for chemical screening. These oncogene transgenic models may be developed to a useful platform in screening of chemicals for discovery of potential drugs to treat liver tumors, particular tumors involving Ras and/or Myc pathways. The feasibility of the high throughput chemical screening is supported by the easy observation and measurement of liver size changes and the possibility to develop an automation system for quantitatively analyzing the changes of liver sizes. While in this study the small molecule inhibitors were added concurrently with oncogene induction for inhibiting carcinogenesis at the initiation stage, it is also feasible to use these inhibitors to treat well-developed tumors in these zebrafish HCC models as we previously reported that some small molecule inhibitors could alleviate the tumor phenotype in \( xmrk \) transgenic zebrafish model\(^{13} \).

In conclusion, our study highlighted the differential requirements of FGF/VEGF, Wnt and Hedgehog signaling pathways in \( kras \)- and \( Myc \)-induced hepatocarcinogenesis. FGF/VEGF signaling is important to both \( kras \)- and \( Myc \)-initiated carcinogenesis while Wnt signaling is critical only to \( Myc \)-induced hepatocarcinogenesis. In contrast, the Hedgehog signaling appeared to be dispensable for both \( kras \)- and \( Myc \)-induced tumors. Effective reduction of \( kras \)- and \( Myc \)-induced liver enlargement and correlated changes of cell proliferation and histopathology suggested that our \( kras^{V12} \) and \( Myc \) transgenic zebrafish models are useful tools for screening of small molecule drugs targeting \( kras \)- and \( Myc \)-induced hepatocarcinogenesis.

Methods

Zebrafish husbandry. All zebrafish experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (Protocol Number: 096/12). Two transgenic lines, \( Tg(fabp10:rtTA2s-M2; TRE2:EGFP-kras^{V12}) \) (gz32Tg) and \( Tg(fabp10:Ta; TRE:myc; CK:RFP) \) (gz26Tg) in a Tet-On system to control the hepatocyte-specific expression of oncogenic \( kras^{V12} \) or \( Myc \) respectively\(^{12,14} \), were used in this study. One reporter transgenic line, \( Tg(fabp10:DsRed; elaA:GFP) \) (gz15Tg) with DsRed-labeled liver and GFP-labeled exocrine pancreas\(^{20} \), was used to either mate with \( Myc \)-expressing transgenic fish to produce offspring with both \( Myc \)- and \( DsRed \)-expressing hepatocytes; or used as negative control.

Chemical treatments. Doxycycline (Dox) (Sigma, D9891) was added from 3 days post fertilization (dpf) to 7 dpf at a dose of 10 \( \mu \)g/ml to induce \( kras \) expression and at 30 \( \mu \)g/ml to induce \( Myc \) expression. SU5402 (Tocris, 3300), SU6668 (tocris 3335), IWR1 (Tocris, 3552), cardionogen 1 (sigma, SML0458), cyclopamine (Tocris, 1825) and GANT61 (Sigma, G9048) were first dissolved in dimethyl sulfoxide (DMSO) as stocks and used for larva exposure from 4 to 7 dpf. The working concentrations used in the experiments were 1 \( \mu \)M SU5402, 1 \( \mu \)M SU6668, 10 \( \mu \)M IWR1, 10 \( \mu \)M cyclopamine and 1 \( \mu \)M GANT61. All of these small molecular inhibitors were previously tested and validated in zebrafish models, such as SU5402\(^{40} \), SU6668\(^{41} \), IWR1\(^{25} \), cardionogene 1\(^{42} \), cyclopamine\(^{43} \) and GANT61\(^{44} \). The dosages were selected based on the highest all-survival concentrations and/or our validation in previous experiments\(^{45,46} \).

Photography and image analysis. At each time point of chemical treatments, 20 larvae of each group were randomly chosen for imaging. The larvae were anesthetized in 0.08% tricaine (Sigma, E10521) and immobilized in 3% methylcellulose (Sigma, M0521). Each larva was photographed separately using an Olympus microscope (DP72). 2D measurement of liver size was performed using ImageJ as previously described\(^{14,47} \).

Histological and cytological analyses. 7 dpf larvae were fixed in 4% paraformaldehyde in phosphate buffered saline (PFA/PBS, Sigma, P6748) and paraffin-sectioned at 5 \( \mu \)m thickness for hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. For IHC staining, rabbit anti-PCNA (Anaspec, AS-55421) primary antibody was used.

Statistics analysis. Statistical analyses were carried out by two-tailed unpaired Student \( t \)-test using inStat version 5.0 software for Windows (GraphPad, San Diego, CA) and data are presented as mean values ± standard error deviation (SED). Throughout the text, figures, and figure legends, \( p < 0.05 \) denotes statistical significance.

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Author Contributions
C.Y. and Z.G. conceived the experiments and wrote the paper. C.Y., Q.Y., X.J.H., H.K.L. and L.Z. performed the experiments. C.Y. analyzed data.

Additional Information
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