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Stimulation of hepatocarcinogenesis by neutrophils upon induction of oncogenic kras expression in transgenic zebrafish

Chuan Yan¹,², Xiaojing Huo¹, Shu Wang¹, Yi Feng¹, Zhiyuan Gong¹,²,

¹Department of Biological Sciences, National University of Singapore, Singapore; ²National University of Singapore Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore; ³MRC Centre for Inflammation Research, Queen’s Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

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Introduction

In the past decade, increasing evidence has indicated a dual role of neutrophils in a variety of tumors [1]. On one hand, activated neutrophils are capable of killing tumor cells through oxidative bursts [2] and secretion of anti-tumor cytokines such as TNF-α and IFNs [3]. On the other hand, in certain situations, neutrophils have also been found to promote tumor progression. Tumor-associated neutrophils (TANs) have long been observed to correlate to tumor progression in chronic colitis-associated carcinogenesis or gastric adenocarcinoma [4–6]. These alternatively behaved neutrophils are capable of releasing growth-stimulating signals, matrix-degrading proteases, and angiogenesis mediators [7], favoring tumor progression. Recently, it has been reported the existence of subtypes N1 (anti-tumoral) and N2 (pro-tumoral) neutrophils; the neutrophil plasticity appears to be regulated by transforming growth factor-beta (Tgf-β), which is often found to be secreted by cancer cells [4]. Neutrophils are induced by Tgf-β to acquire an N2 phenotype, which differs from N1 neutrophils that require inhibition of Tgf-β and sufficient ifn-β [4,8]. The influence between neutrophils and cancer cells are reciprocal. While tumor cells are capable of hyper-expressing pro-inflammatory molecules, mimicking the initial phase of wound healing [9], to attract neutrophils to the localized tumor microenvironment [10], the infiltrating neutrophils also have pro-angiogenic effects and promotes epithelial mesenchymal transition during tumor progression [11,12]. This integral relationship between immune cells and tumor cells is particularly evident in HCC, which is a typical inflammation-associated cancer since the primary etiological factors, hepatitis B and C viruses, create an unresolved, chronic inflammation of the liver [13]. Thus, immune-based therapy became a new promising approach for HCC patients. The study of the interaction between HCC and neutrophils should provide much needed insights into the development of such a therapeutic approach.
Recently we have generated several inducible HCC models by transgenic expression of an oncogene in zebrafish hepatocytes [18–21]. A major advantage of these inducible models is the temporal control of cancer initiation to provide an excellent opportunity to characterize tumor initiation events, hitherto lacking in human clinical studies and other non-inducible tumor models. Furthermore, the transparency of zebrafish larvae allows us to monitor early in vivo hepatocarcinogenesis and progression, thus providing a plethora of opportunities to investigate the initiation events in hepatocarcinogenesis and the roles of various cancer hallmark factors in the process. In particular, we recently observed a prominent immune response in HCC progression and regression in one of our zebrafish HCC models based on RNA-Seq analyses [22]. In the present study, the interaction of neutrophils and oncogenic hepatocytes in hepatocarcinogenesis was investigated. We observed a rapid recruitment of neutrophils into oncogenic liver, which led to accelerated tumor progression. Molecular analyses of fluorescence-activated cell sorting (FACS)-isolated hepatocytes and TANs indicated changes of several important molecular pathways, including promotion of a pro-inflammatory microenvironment in oncogenic hepatocytes and decreases of anti-tumor in TANs. Thus, our data suggest a promoting role of neutrophils in early hepatocarcinogenesis.

Materials and methods

Zebrafish husbandry

Zebrafish were maintained in compliance with Institutional Animal Care and Use Committee guidelines, National University of Singapore. Four transgenic lines, Tg(fabp10:rtTA2s-M2; TRE2:EGFP-krasG12V) [18], Tg(lyz:DsRed2)xf0 with DsRed-labeled neutrophils under the lysozyme C (lyz) promoter [23], Tg(fabp10:DsRed; ela3l:GFP)ls2 with DsRed-labeled liver and GFP-labeled exocrine pancreas [24], Tg(mpeg1:GFP)y2j, with GFP-labeled macrophages under the mpeg1 promoter [25], were used and referred to as kras, lyz, fabp10, and mpeg, respectively, in this report.

Chemical treatment

20 μg/ml doxycycline (Sigma, D9891) was added from 3 days post-fertilization (dpf) to 8 dpf to induce krasG12V-EGFP expression. Lipopolysaccharides (LPS) (Sigma, L4391), FPR-A14 (Tocris, 2826), PR-39 (Tocris, 1947) and SB431542 (Tocris, 1614) were first dissolved in dimethyl sulfoxide as stocks and used for larva exposure at 5 ng/ml, 2.5 μM, 50 nM and 2.5 μM, respectively from 4 to 8 dpf. The dosages were selected based on the highest all-survival concentrations.

Statistical analysis

Statistical significance between two groups was evaluated by Student’s t test using inStat version 5.0 for Windows (GraphPad, San Diego, CA). Student’s t-test was used to calculate probability (p values) of different groups in comparison. Throughout the text, the word “significance” is used to denote statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

Other methods

Other methods are described in Supplementary Materials and methods, including morpholino knockdown and Tgf-β depletion; photography and image analysis; isolation of hepatocytes and neutrophils by FACS; RNA extraction, cDNA amplification and RT-qPCR (reverse transcription-quantitative PCR); and histological and cytological analyses.

Results

Rapid recruitment of neutrophils into krasV12-expressing livers

To visualize the inflammation response, hemizygous kras+ transgenic fish were crossed with lyz+ homozygous fish for production of kras+ and lyzw– offspring in the lyz+ background. DsRed expressing neutrophils were monitored up to 96 hpi (hour post-induction with doxycycline). As shown in Fig. 1A and quantified in Fig. 1B–D, the total counts of neutrophils in the liver region was noticeably increased from as early as 8 hpi and became statistically significant from 16 hpi (Fig. 1B). These neutrophils within the vicinity of the liver, considered as TANs, were normalized against the liver size as neutrophil density. As shown in Fig. 1C, a significant increase in neutrophil density was observed from 8 hpi; thus, neutrophils were actively recruited to the site of tumor initiation within 8 hours of oncogene activation. In contrast, the increase of liver size became apparent only from 24 hpi (Fig. 1D), indicating that neutrophil recruitment preceeded liver enlargement. To further evaluate the contribution of neutrophils to the increased liver size in kras+ larvae, neutrophil density and liver size were plotted for each individual kras+ larva and we observed a strong positive correlation (Pearson’s coefficient, 0.62); in contrast, such a correlation was not present in kras– siblings (Pearson’s coefficient, 0.20) (Fig. 1E).

Acceleration of hepatocarcinogenesis by stimulation of general immune response and neutrophils

To further demonstrate the role of inflammatory cells in initiation and progression of hepatocarcinogenesis, we first tested a general inflammatory stimulator, LPS, which has been demonstrated to stimulate the immune system in zebrafish larvae [26]. 5 ng/ml of LPS was used to treat zebrafish larvae from 4 dpf to 8 dpf. kras+ larvae exposed to both LPS and doxycycline showed significant increases of both neutrophil count and density in the liver as compared to kras+ larvae exposed to doxycycline alone and all kras– groups. Interestingly, there was also a further enlargement of liver size with the increased neutrophils (Fig. 2A). To investigate if the accelerated liver enlargement was indeed associated with increased neutrophil infiltration, FPR-A14, which is a formyl peptide receptor agonist and has been reported to potently activate neutrophils specifically in vitro [27], was used to challenge the kras+ larvae from 4 dpf to 8 dpf. Liver neutrophil count and density in FPR-A14 and doxycycline double-treated kras+ larvae were also significantly higher than those of their kras+ sibling treated with only doxycycline and of all kras– groups (Fig. 2B), similar to that observed following LPS treatment. A further liver enlargement was also observed from these double-treated kras+ larvae. To further demonstrate the effect of neutrophils, kras+ transgenic larvae were also challenged with a neutrophil inhibitor, PR-39, a proline-rich anti-bacteria peptide that inhibits NADPH oxidase activity in neutrophils [28]. As shown in Fig. 2C, liver neutrophil count and density as well as liver size in kras+ larvae exposed to PR-39 and doxycycline were all decreased as compared to kras+ sibling controls treated with doxycycline alone and all kras– groups. Thus, there was a good correlation between numbers of infiltrated neutrophils and the size of oncogenic liver, suggesting an in vivo promoting role of neutrophils in early hepatocarcinogenesis.
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Fig 1. Recruitment of neutrophils to oncogenic liver. (A) Representative images of kras−/lyz− and kras+/lyz+ larvae after 8, 24, and 96 hours of doxycycline induction starting from 3 dpf. The livers are outlined in blue dash lines in the upper images of kras−/lyz− larvae and marked by GFP expression in the lower images of kras+/lyz+ larvae. (B–D) Time course of neutrophil count (B), neutrophil density (C) and liver size (D) following induction of oncogenic krasV12+ expression in hepatocytes (n >15 from each group). Neutrophils were counted in the liver area and normalized against the liver size for liver density. Liver size was measured based on 2D images. (E) Correlation of liver size and neutrophil density in the liver. The measurements were based on 8 dpf larvae after 5 days of doxycycline induction. A positive correlation was observed only in the kras+ transgenic larva (top) but not in the kras− control group (bottom). Statistical significance: * p <0.05, ** p <0.01, *** p <0.001.

Fig. 2. Effect of infiltrated neutrophils on liver size. (A–C) Neutrophil counts (left) and density (middle) in the liver area and liver size (right) in response to treatments of LPS (A), FPR A14 (B) or PR39 (C). Both kras−/lyz− and kras+/lyz+ groups were similarly exposed to doxycycline from 3 dpf and neutrophils mediators were added from 4 dpf. Neutrophils and liver sizes were determined at 8 dpf (n >15 from each group). Statistical significance: p <0.05.

Inhibition of neutrophil differentiation defers HCC progression

To further validate the effect of neutrophils on tumor growth, differentiation of myeloid derived precursor cells into neutrophils was blocked via morpholino knockdown of the gcsfr gene (MO_gcsfr). MO_gcsfr or control morpholino (MO_SC) were injected into lyz+ embryos at one-cell stage and injected embryos were monitored for DsRed+ neutrophils. As shown in Supplementary Fig. 1A and C, by 6 dpf, there was an overall decrease of circulating neutrophils in MO_gcsfr injected larvae, compared to those in lyz+ larvae injected with MO_SC, consistent with the previous report that used the same set of morpholinos [29]. kras+/lyz+ and kras−/lyz+ zebrafish embryos were then injected with MO_gcsfr and analysed at 6 dpf after doxycycline induction from 3 dpf. Similar to the observations with the PR-39 inhibitor, a significant decrease of neutrophil counts and density in the liver (Fig. 3D and E) as well as liver size (Fig. 3F) was observed, further confirming a promoting role of neutrophils in the initial stage of hepatocarcinogenesis.

To evaluate the role of macrophages in our model, depletion of macrophages was carried out by morpholino knockdown of the irf8 gene [25]. As shown in Supplementary Fig. 1B and C, when mpeg+ embryos were injected with MO_irf8, GFP expressing macrophages were greatly reduced; meanwhile, there was a compensating increase of neutrophils, consistent with a previous report that knockdown of irf8 causes the common progenitor cells to differentiate to neutrophils [25]. When MO_irf8 was injected into kras+/lyz+ embryos, we observed a significant increase of neutrophil counts and density in the liver.
Table 1: Neutrophil subpopulations and their effects on liver size.

<table>
<thead>
<tr>
<th>Neutrophil Subpopulation</th>
<th>Effects on Liver Size</th>
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<tr>
<td>Normal</td>
<td>Increased</td>
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<tr>
<td>Tumor-Infiltrated</td>
<td>Increased</td>
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<tr>
<td>Macrophage-Dominated</td>
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Discussion

The results of this study suggest that neutrophils play a critical role in the development of hepatocarcinogenesis. The increase in segmented nuclei of TANs, as compared to NNs, indicates a higher degree of differentiation and maturation of these neutrophils. This is supported by the increase in apoptosis in TANs, which is typical of mature neutrophils.

Promotion of proliferation and suppression of apoptosis in krasG12V-expressing hepatocytes by infiltrated neutrophils

To identify the causative factors for liver size change when neutrophil activity was modulated, hepatocyte proliferation and apoptosis were examined. As shown in Fig. 5, there were significant increases of hepatocyte proliferation and apoptosis in doxycycline-induced kras+ larvae, suggesting highly aberrant cell division and death in oncogenic livers. While co-exposure to
FPR-A14/doxycycline did not induce a further increase of proliferating hepatocytes, it did lead to a significant reduction of apoptotic hepatocytes. In contrast, co-treatment of kras+ larvae with PR-39/doxycycline reduced the number of proliferating hepatocytes and increased the number of apoptotic hepatocytes. Thus, the changes in neutrophil activity affect both proliferation and apoptosis of hepatocytes.

Moderated histological phenotype of oncogenic hepatocytes by inhibition of neutrophil activity

Histologically, as shown in Fig. 6A, all H&E staining of 8 dpf kras+ larvae exposed to doxycycline showed histological features of early carcinoma, e.g. disorganized cell plate, large nucleus-to-cytoplasm ratio and pseudo glandular patterns. All kras+ larvae exposed to FPR-A14 and doxycycline showed similar early carcinoma histology, but with significantly less proportion of normal tissue, suggesting that the additional exposure to FPR-A14 had further stimulated HCC progression. In contrast, most kras+ larvae exposed to PR-39 and doxycycline did not display the early carcinoma phenotype and instead resembled closely to the liver phenotype of control kras− larvae, implying exposure to PR-39 deters progression into HCC. Fig. 6B summarizes the quantitative distribution of histological phenotypes in each treatment group, further indicating that inhibition of neutrophils led to moderated tumor phenotype while activation of neutrophils deteriorated the phenotype. The liver phenotype was also examined by immunostaining using two fibrosis markers, Laminin and Collagen 1a [30] (Supplementary Fig. 2) and we observed increases of both biomarkers in induced kras+ oncogenic livers, which were further increased by FPR-A14 but attenuated by PR-39, consistent with the general trends of liver tumor severity based on H&E staining.

Pro-inflammatory microenvironment of oncogenic liver and reduction of anti-tumor activities of TANs

It has been well established that cancer cells are capable of creating a pro-inflammatory microenvironment [31,32]. To investigate molecular interaction of oncogenic hepatocytes and...
neutrophils in our model, GFP-krasG12V-expressing hepatocytes were isolated from doxycycline-treated kras+ larvae by FACS and meanwhile enriched TANs were isolated based on DsRed expression (Fig. 7A). RT-qPCR analysis of kras+ hepatocytes (Fig. 7B) showed a significant upregulation of tgfβ1a, a primary regulator in early development of liver fibrosis and cancer-related inflammation [33,34]. In contrast, key anti-tumor genes such as il6 and il4 showed significant downregulation. Meanwhile, compared to NNs, TANs showed a general pro-tumor gene expression pattern. For instance, il1b, which promotes early cancer angiogenesis [35], was significantly upregulated while anti-tumor cytokines, il4, il6, il8, il10, il12, and tnfa, all showed significant downregulation (Fig. 7C). Tgfβ-1 is known to polarize neutrophils to a pro-tumor phenotype [4]. To investigate if oncogenic hepatocyte-secreted Tgf-β mediated crosstalk between the two cell types, SB431542, a specific inhibitor of Tgf-β type I receptor [36,37], was used to block Tgf-β signaling in TANs where both tgfβ1a and tgfβ1b were found to be expressed (data not shown). Compared to unblocked TANs, significant upregulation of all but one examined anti-tumor cytokines (il6, il8, il10, il12, and tnfa) was observed in Tgf-β receptor blocked TANs. Thus, blockage of Tgf-β signaling partially rescued the expression of these anti-tumor genes (Fig. 7D).

Since Tgf-β has been reported to be a chemoattractant for neutrophils [38], we further depleted Tgf-β by injection of zebrafish Tgf-β antibody into 3 dpf kras+/lyz+ zebrafish as well as by SB431542 inhibition of Tgf-β receptor [39]. We noticed reduced neutrophils in the liver by 8 dpf by both approaches (Fig. 8A and C) as confirmed by the decreases in neutrophil numbers and density in the liver, which was also accompanied with a decrease in liver size (Fig. 8B and D). The successful depletion of Tgf-β was supported by significant reductions of phosphorylated Smad2, a downstream marker of the Tgf-β pathway in both neutrophils and hepatocytes (Fig. 8E–F). Collectively, these experiments demonstrate the role of Tgf-β in attracting neutrophils to the liver upon oncogenic kras activation in hepatocytes.

Discussion

Rapid response of neutrophils to a pro-inflammatory microenvironment created by oncogenic krasV12-expressing hepatocytes

The chronic, unresolved inflammation is well recognized as one of the hallmarks and a contributing factor of early HCC. Persistent inflammation of the liver drives hepatocyte apoptosis and compensatory proliferation, while prolonged abnormal regeneration potentially promotes hepatic damage, fibrosis, cirrhosis and eventually HCC [40]. TGF-β plays a crucial role in the progression of the liver disease as the initial damage of liver upregulates TGF-β to lead to a wound healing response [41]. In our krasG12V transgenic model, krasG12V-expressing hepatocytes displayed an upregulation of tgfβ1a and downregulation of anti-tumor genes, tnfa and il1b. The gene expression pattern indicated that the oncogenic kras+ hepatocytes favored a pro-inflammatory environment, consistent with a previous report of liver tumors arising from inflammation due to chronic injury [42]. Moreover, decreased expression of tnfa and il1b aids in the creation of a pro-tumor microenvironment, which is in line with clinical HCC data [43,44]. Thus, oncogenic krasG12V expression have created a pro-inflammatory microenvironment in the HCC initiation stage of HCC and downregulation of important anti-tumor genes further facilitates the disease progression. Moreover, TAN showed significant downregulation of a variety of anti-tumor cytokines, including il4, il6, il8, il10, il12, and tnfa, and upregulation of pro-tumor il1b. These observations indicate a crosstalk between the oncogenic hepatocytes and neutrophils. Oncogenic hepatocytes secrete high level of Tgf-β which recruit neutrophils as it has been demonstrated to be a potent chemoattractant of neutrophils [38]. We further validated that depletion of Tgf-β resulted in a significant reduction of neutrophil density in the oncogenic livers, which caused a decrease of oncogenic liver size compared to oncogenic liver without Tgf-β depletion (Fig. 8D). However, the liver size remained significantly larger than the size of livers in kras+ controls, which may indicate multiple roles of Tgf-β, including a suppressor in early tumor progression [45]. Once the neutrophils are recruited, a high level of Tgf-β in oncogenic hepatocytes further causes TANs to reduce expression of anti-tumor genes and increase in il1b. Increased expression of il1b in TANs hints the possible angiogenesis promoting
The role of TAN in HCC. Blockade of the TGF-β signaling pathway by SB431542 led to a partial rescue of the pro-tumor gene expression profile in TAN, further validating the molecular crosstalk between the two cell types.

Acceleration of HCC progression by TANs

Our data indicated a good correlation between the number/density of liver-infiltrated neutrophils and the size of oncogenic liver; thus, it is likely that neutrophils play a stimulating role in liver tumor initiation and progression. This phenomenon has been further confirmed by pharmacological experiments. Both the general immune stimulator LPS and neutrophil-specific activator FRP-A14 caused further increase of liver-infiltrated neutrophils and also further increase of the liver size. Conversely, neutrophil inhibitor, PR-39, caused a reduction of liver-infiltrated neutrophils and also slowed down the growth of oncogenic liver. The observed effects by FRP-A14 and PR-39 appear to be mainly due to the change of neutrophil numbers in the liver as neither chemical affects the number of macrophages in the liver (Supplementary Fig. 3). In addition, using Irf8 morpholino to deplete macrophages and thus increase neutrophil density, we demonstrated that neutrophil alone also have a significant effect in accelerating HCC progression.

Fig. 8. Effect of antibody-mediated TGF-β depletion on TAN recruitment. (A) Representative images of 8 dpf kras+/lyz+ larvae exposed to either doxycycline alone (top) or with SB431542 (bottom). (B) Neutrophil counts (left) and density (middle) in the liver and liver size (right) after SB431542 treatment. (C) Representative images of 8 dpf kras+/lyz+ larvae injected with TGF-β antibody (bottom) or mock injected as a control (top). Both were induced by doxycycline. (D) Neutrophil counts (left) and density (middle) in the liver and liver size (right) in TGF-β-depleted (tgfb-1D) and mock injected (MI) larvae. (E) Validation of SB431542 exposure and TGF-β depletion by immunostaining of Smad2 and phospho-Smad2 (pSmad2). Representative liver sections are shown from each group as indicated. The color code of each probes correspond the color signals in the images. Red arrows, DsRed expressing neutrophils with pSmad2 expression; white arrows, DsRed expressing neutrophils without pSmad2 expression. (F) Ratios of pSmad2/sm22 in neutrophils (top) and hepatocytes (bottom) in SB431542-inhibited and TGF-β-depleted larvae. n = 10; *p < 0.05.
Histologically, the severity of oncogenic livers also correlated to the activity of neutrophils. There was a rapid oncogenic transformation of hepatocytes upon kras<sup>G12V</sup> induction and histological HCC phenotype could be observed within four days of doxycycline induction. Neutrophils likely play an important role in this process as inhibition of neutrophils by PR-39 resulted in a liver histology close to a normal liver. The possible explanation of the neutrophil-accelerated tumorigenesis lies in the ability of neutrophils to induce proliferation and inhibit apoptosis in oncogenic hepatocytes. Enhancement of the neutrophil activity using an activator showed no further increase in hepatocyte proliferation but significant inhibition of apoptosis. In contrast, inhibition of neutrophil activity with PR-39 led to inhibition of proliferation and promotion of apoptosis in hepatocytes (Fig. 5B and D).

Characteristics and behavior changes of neutrophils in tumor microenvironment

It has been well documented that neutrophils are a heterogeneous population and the characteristic and behavior of TANs and non-infiltrated neutrophils are markedly different [4]. In our study, we also noticed several major differences between TANs from oncogenic kras+ larvae and NNs from kras− control larvae. First, migratory patterns of TANs appear to be relatively active when the cells were meandering along the tumor perimeter, and become relative motionless in the tumor, while the migration of NNs in kras− larvae are uniformly active regardless of their locations. Second, Giemsa staining of TANs showed a high percentage of hyper-segmented nuclei, which is consistent with the report that pro-tumor N2 neutrophils in mouse models have hyper-segmented nuclear structure while anti-tumor N1 neutrophils have largely round nucleus morphology [4]. Third, by RT-qPCR analyses, TANs showed downregulation of anti-tumor genes (e.g., il4, il6, ilb, il10, il12, and infa) and upregulation of pro-tumor genes such as il1b, which also promotes early cancer angiogenesis [35], indicating a potential role of TANs in pro-angiogenesis in HCC initiation. A complete search for more critical genes expressed in TANs for promoting early hepatocarcinogenesis could be carried out in the future by RNA-Seq analyses in our present model.

In summary, our study suggests that oncogenic kras<sup>G12V</sup> expression in hepatocytes favors a pro-inflammatory microenvironment by increased Tgf-β1α expression, which attracts a rapid recruitment of neutrophils to oncogenic livers. These infiltrated neutrophils have stimulating roles in early hepatocarcinogenesis and they lost anti-tumor activity. Our data are consistent with the view that the presence of neutrophils in the tumor microenvironment is an important marker in the aggressiveness of the liver cancer progression [46,47]. A high neutrophil density in the tumor would be an indicator of high hyperplasia and low apoptosis. Thus, Neutrophils are not bystanders in hepatocarcinogenesis and instead they are actively promoting its initiation and progression.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors’ contributions

Conceived and designed the experiments: CY, XH, SW, YF, ZG. Performed the experiments: CY, XH. Analyzed the data: CY, XH, ZG. Contributed reagents/materials/analysis tools: SW, YF. Wrote the paper: CY, XH, ZG.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.03.024.

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