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Hepassocin is required for hepatic outgrowth during zebrafish hepatogenesis

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ABSTRACT

Background & aims: Hepassocin (HPS) is a hepatotrophic growth factor that specifically stimulates hepatocyte proliferation and promotes liver regeneration after liver damage. In this paper, zebrafish were used to investigate the role of HPS in liver development.
Methods and results: During zebrafish development, HPS expression is enriched in liver throughout hepatogenesis. Knockdown of *HPS* using its specific morpholino leads to a smaller liver phenotype. Further results showed that the *HPS* knockdown has no effect on the expression of the early endoderm marker *gata6* and early hepatic marker *hhex*. In addition, results showed that the smaller-liver phenotype in HPS morphants was caused by suppression of cell proliferation, not induction of cell apoptosis.
Conclusions: Current findings indicated that *HPS* is essential to the later stages of development in vertebrate liver organogenesis.

1. Introduction

Hepassocin (HPS), also called FGL1 and hepatocyte-derived fibrinogen-related protein (HFREP-1), is mostly expressed in liver and has mitogenic activity on isolated hepatocytes [1–3]. HPS belongs to fibrinogen superfamily which contains an N-terminal signal recognition peptide, a potential N-terminal coiled-coil domain, and a C-terminal fibrinogen related domain [1,4,5]. The cytokine HPS stimulates the proliferation of primary hepatocytes and normal hepatocyte lines by binding to specific receptors expressed on hepatocyte cell surface but does not promote DNA synthesis in non-hepatic-derived established cell lines *in vitro* [3]. Although there is a basal level of HPS expression in mouse livers, HPS is highly up-regulated after 70% hepatectomy [1,2]. In addition, IL-6 has been found to induce HPS expression in HepG2 hepatoma cells in a dose-dependent manner [6]. Previous studies have shown that HNF1α is critical to liver-specific expression of HPS [6]. It has been demonstrated that administration of recombinant HPS significantly protects rats from chemical-induced hepatitis injury [2]. These data suggest that HPS functions as a regulator of cell growth in liver regeneration and may protect hepatocytes from injury. Further studies showed that the expression of the *HPS* was frequently down-regulated or lost in HCC tissue [7]. Overproduction of truncated HPS, whose signal peptide was deleted, significantly inhibited the proliferation of HCC cells and induced cell cycle arrest, suggesting that HPS inhibits HCC cell proliferation through an intracrine pathway [3]. Recent studies have shown that gene ablation of HPS in mice results in abnormal plasma lipid profiles, fasting hyperglycemia with enhanced gluconeogenesis, and differences in white and brown adipose tissue morphology [8], indicating that HPS is a member of an emerging group of proteins that have key roles in metabolism. Studies have provided evidence

Abbreviations: GATA6, GATA-binding factor 6; Hhex, hematopoietically-expressed homeobox protein; FGL1, Fibrinogen-like 1; IL-6, interleukin- 6; HNF1α, hepatocyte nuclear factor1α; HCC, Hepatocellular Carcinoma; Fabp10, fatty acid-binding protein 10.

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that HPS plays an important role in non-alcoholic fatty liver disease (NAFLD) and induces hepatic lipid accumulation through an ERK1/2-dependent pathway [9].

Zebrafish (*Danio rerio*) is an excellent vertebrate model to study embryonic liver development. Liver development in zebrafish is similar to that in mammals and other vertebrates but they do not generally experience hematopoietic defects [10–12]. In mammals, HPS signaling was found to promote hepatocyte proliferation, protect hepatocytes from injury, and regulate lipid metabolism [2,8,9]. However, the function of HPS in zebrafish livers is not yet well understood. In this study, zebrafish were used to investigate the role of HPS in liver development. We report here that HPS was mostly expressed in the developing livers of zebrafish, and morpholino (MO) knockdown of HPS reduced the size of the developing liver through the suppression of cell proliferation, not enhancement of cell apoptosis. These data indicate that HPS is essential to hepatic outgrowth.

2. Materials and methods

2.1. Zebrafish lines

The fabp10 transgenic line, *Tg(fabp10:RFP)* zebrafish was a gift from prof. Qiang Wang in The Chinese Academy of Sciences Institute of Animal (Beijing, China). The Tuebingen AB line of zebrafish was used as wild-type. Embryos were collected from natural mating and raised in Holtfreter's solution at 28.5 °C, and staged morphologically as described previously [13]. All experiments were conducted in accordance with the guidelines approved by the committee on animal care at Beijing Institute of Radiation Medicine.

2.2. Morpholino oligonucleotides (MO)

Morpholino oligonucleotides (MO) for *HPS* splice donor (UTR-MO) (5′-GAA AAC TAT TGC TAA CCT TCG AAC C-3′) was designed to be complementary to the mRNA guide strand according to the sequence of the HPS gene in zebrafish. The standard control morpholino from GeneTools served as a non-specific control. MOs were synthesized using Gene Tools (Philomath, U.S.) and diluted with nuclease-free water. Here, 1 nl morpholino (4 or 8 μg/ml) was injected into 1- to 4- cell embryos.

2.3. mRNA rescue

The full coding sequence of HPS was amplified from the zebrafish cDNA using the forward primer (5′-AAT GAT ATC ATG CCA CAG CTG GTG TT-3′) and the reverse primer (5′-CCG CTC GAG CTA TTC AGT GGG CCC TCC TCC-3′) and then inserted into the EcoR V and Xho1 sites of the pCS-Flag vector. HPS mRNA was obtained from pCS-Flag-HPS via *in vitro* transcription using a Message Machine Kit (Ambion). In the mRNA rescue assay, 200 ng HPS mRNA and 8 ng MOs were injected into 1- to 4-cell embryos.

2.4. RNA extraction and RT-PCR

Total RNA was extracted from embryos at different developmental stages using TRIzol reagent (Invitrogen) following manufacturer's instruction. Zebrafish HPS cDNA was obtained using two-step RT-PCR kit (Promega). The forward primer (5′-ATG GTT GTC TTT ACA AGC GGT G-3′) and the reverse primer (5′-AAG ATC CTC CAG TGC TTG GT-3′) were used to test the effect of HPS morpholinos.

2.5. Whole-mount in situ hybridization (WISH)

Digoxigenin-UTP-labeled antisense RNA probes were *in vitro* transcribed using an *in vitro* transcription kit (Promega) according to the manufacturer's instructions. The probes against *hhex*, *fabp10*, *insulin*, and *gata6* were provided by Prof. Qiang Wang of the Chinese Academy of Sciences Institute of Zoology (Beijing, China). The forward primer (5′-TTG TAT ACT GTG ACA TGG AT-3′) and the reverse primer (5′- TCC ACC TCT GTA AAA TCT CC-3′) were used to amplify the sequences for digoxigenin-labeled RNA antisense probe of *HPS*. Whole-mount in situ hybridization was performed as described previously [14,15].

2.6. Immunohistochemistry for proliferation and TUNEL assay

Embryos were fixed in 4% paraformaldehyde overnight at 4 °C and then embedded and sectioned as described [2]. For the proliferation assay, the sections were incubated with rabbit anti-phospho histone H3 (p-H3) antibody (1:100 dilution, Santa Cruz) overnight at 4 °C. Secondary antibodies of HRP-conjugated anti-rabbit IgG (Cell signaling technology) were then incubated for 1 h at room temperature. TUNEL assay was performed using a Roche in situ cell death detection kit in accordance with the manufacture's instructions. An immunohistochemistry assay was performed as described previously [16]. The pictures were imaged with a Nikon microscope.

2.7. Statistical analysis

All results are expressed as means ± SD, and the statistical significance was assessed by one-way analysis of variance (ANOVA) and Student t tests. P values <0.05 were considered statistically significant.

3. Results

3.1. HPS expression is enriched in liver tissue

By searching an adult zebrafish EST database (GenBank, National Center for Biotechnology Information), a sequence (fibrinogen-like-protein 1 gene, GenBank accession number XM_679486) possessing striking similarities to human and mouse HPS was found. The full-length coding sequence of *zebrafish* HPS was obtained by RT-PCR. The full-length coding sequence was 1008 bp, and the deduced amino acid sequence of the *zebrafish* HPS gene encoded 336 amino acids. The HPS protein sequence is highly conserved between zebrafish and mammals: human and zebrafish proteins share 46% identity overall (44% identity between zebrafish and mice), and 47% identity in the N-terminal signal recognition peptide and 55% identity in the C-terminal fibrinogen related domain (Fig. S1). Alignment of transcribed sequences with their respective genomic DNA sequence reveals that the zebrafish *HPS* has genomic structures similar to those of the human and mouse HPS genes (Fig. S2). In addition, zebrafish HPS also contains five conserved cysteine residues. These are used to form a homo or heterodimer through S^S bridges in human and mouse HPS (data not shown). Phylogenetic analysis using MEGA6 software showed that HPS is well conserved during vertebrate development (Fig. 1A), and HPS in zebrafish is closely related to Osteichthyes such as *Osmerus mordax* and *Oryzias latipes* but displays a relatively large evolutional distance from mammals such as humans and mice.

To understand the expression of HPS during zebrafish liver development, a digoxigenin-labeled RNA antisense probe for *HPS* was used to detect the expression of HPS. As shown in Fig. 1B, HPS mRNA was overwhelmingly expressed in the developing liver from the liver budding stage (48 hpf) and intensified during the liver

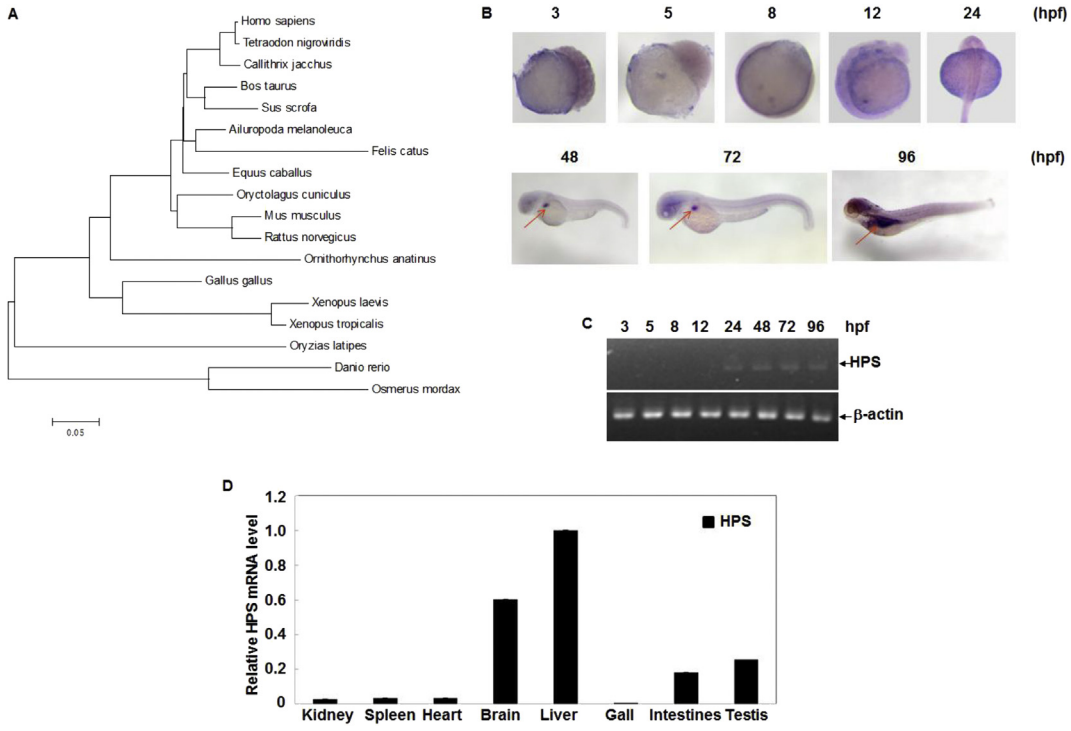

Fig. 1. Expression and phylogenic tree analysis of zebrafish HPS. (A) Phylogenic tree of HPS family. (B) Lateral and dorsal view of restricted expression of HPS mRNA at different embryonic stages through whole-mount in situ hybridization (WISH) assay. (C) RT-PCR analysis of HPS mRNA at different embryonic stages, β-actin served as a control. Red arrows indicate HPS in the developing liver. (D) RT-PCR analysis of HPS mRNA levels in different tissues of adult zebrafish, endogenous β-actin gene was used to normalize RT-PCR data.

growth phase (96 hpf). The mRNA expression of HPS was confirmed by RT-PCR assay (Fig. 1C). The highly specific HPS expression in the developing liver throughout hepatogenesis suggest that HPS might play an important role in liver organogenesis. In addition, RT-PCR revealed that HPS is specifically expressed in liver tissue in adult fish which is similar to that of the mouse and human HPS (Fig. 1D).

3.2. Knockdown of HPS with MOs results in small liver phenotype

To study the function of HPS in liver development, one morpholino targeting exon1-intron1 boundary (E1 MO) was designed (Fig. S3). Morpholinos were microinjected into 1- to 4-cell embryos using standard control morpholinos as controls. To confirm the effects of E1 MO, con MO and HPS E1 MO were injected into embryos at the 1- to 4-cell stage. RT-PCR showed that the injection of E1 MO resulted in the production of aberrantly spliced mRNA, but no such alternate splices formed in control-injected embryos, demonstrating accurate targeting of the morpholinos. The efficacy of the E1 MO could be demonstrated by RT-PCR results showing significantly reduced levels of endogenous *HPS* transcript (Fig. 2A). These results demonstrated that HPS E1 MO is specific and effective for knockdown of the expression of HPS.

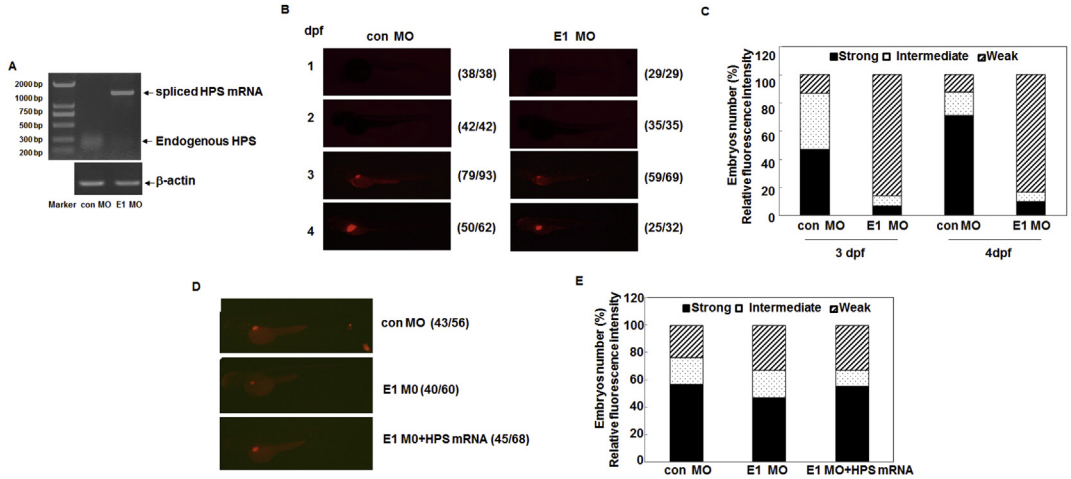

Fig. 2. HPS E1 MO and small liver phenotypes. (A) RT-PCR analysis HPS pre-mRNA expression at 24 hpf when embryos were injected with 8 ng con MO or 8 ng E1 MO. (B, C) *Tg(fabp10: RFP)* transgenic embryos were microinjected with 8 ng con MO or 8 ng E1 MO, respectively, then fluorescence was visualized under a Nikon fluorescence microscope and relative fluorescence intensity in the liver was calculated at 1–4 dpf. (D, E) *Tg(fabp10: RFP)* transgenic embryos were injected with 8 ng con MO, 8 ng E1 MO or combination of 8 ng E1 MO with 200 pg HPS mRNA, fluorescence was visualized under a Nikon fluorescence microscope and relative fluorescence intensity in the liver was calculated at 3 dpf.

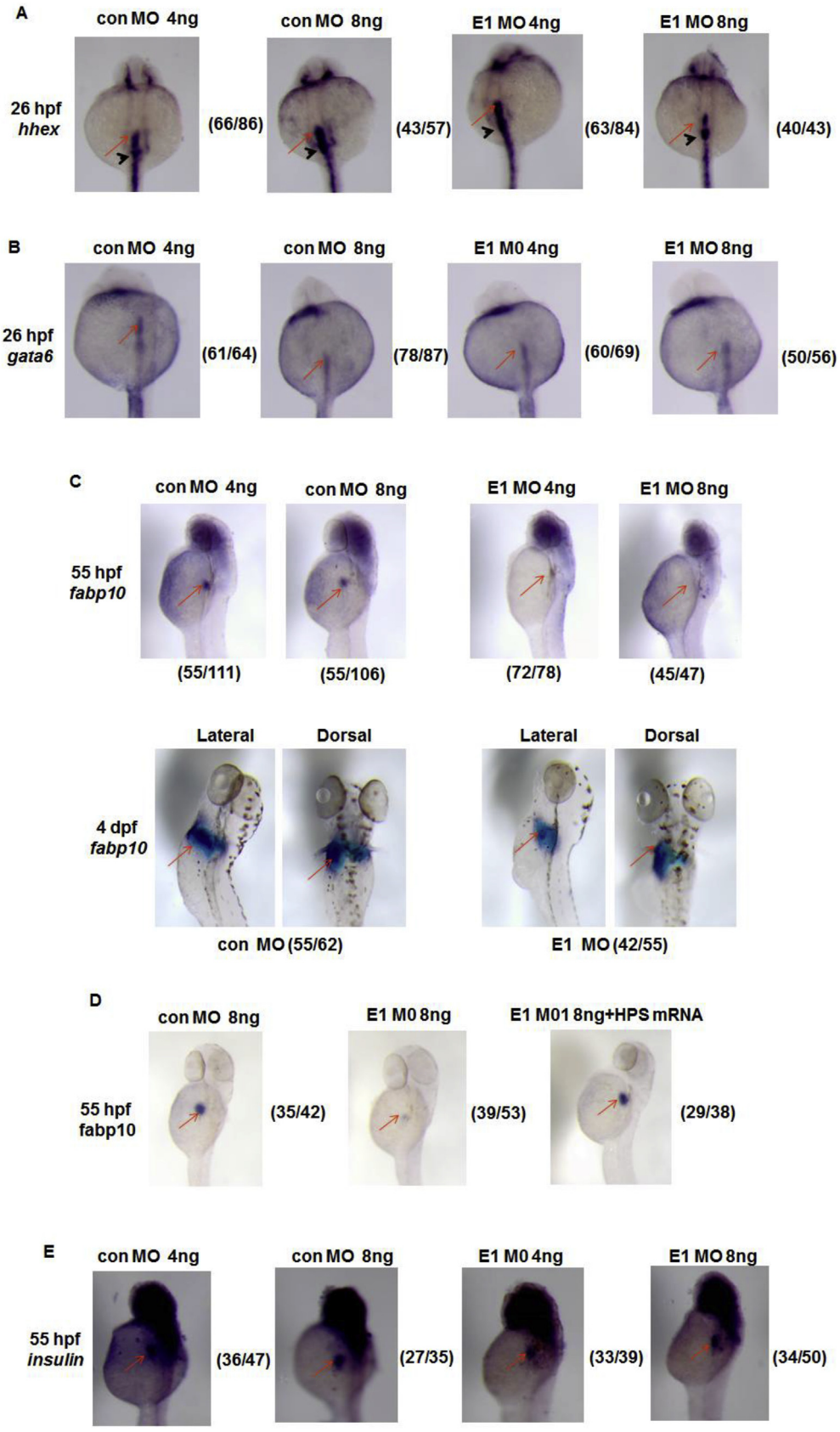

Fig. 3. HPS depletion regulates liver outgrowth but not liver specification and budding in zebrafish. (A) AB zebrafish embryos were injected with 4/8 ng con MO or E1 MO. Then WISH assay was performed to detect the mRNA expression of *hhex* at 26 hpf. Red arrows indicate the liver and black arrows indicate the pancreas. (B) Embryos were injected with 4/8 ng con MO or E1 MO. Then the WISH assay was performed to detect the mRNA expression of *gata6* at 26 hpf. Red arrows indicate endoderm. (C) Embryos were injected with con MO and E1 MO. Then the WISH assay was performed to detect the mRNA expression of *fabp10* at 55–96 hpf. Red arrows indicate the liver. (D) Embryos were injected with 8 ng con

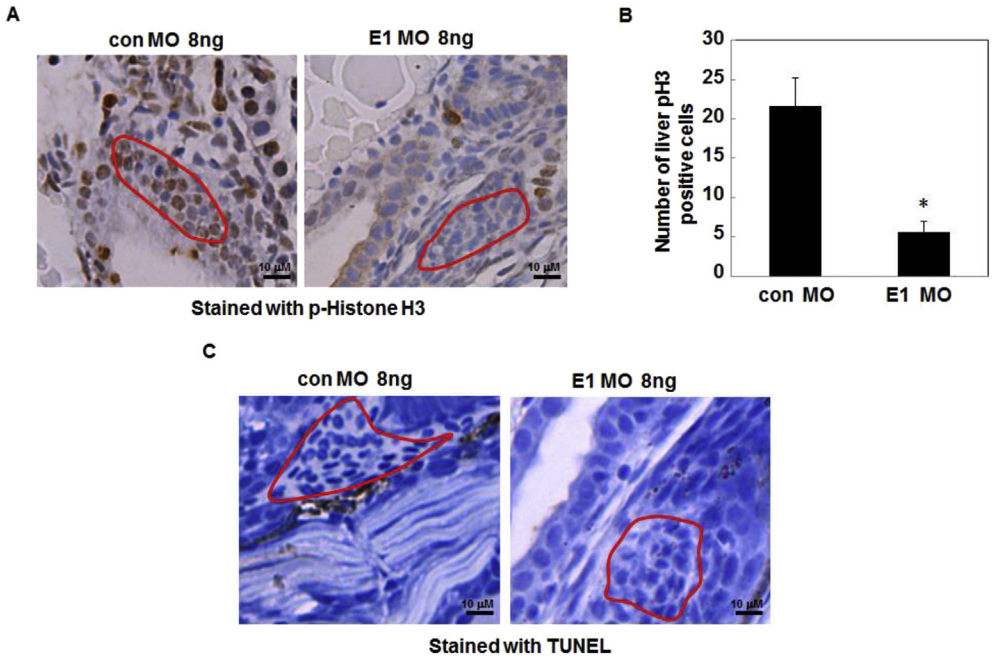

Fig. 4. HPS knockdown, hepatocyte proliferation, and apoptosis. (A, B) Embryos were injected with 8 ng con MO or E1 MO. Then an immunohistochemistry assay involving staining with cell proliferation marker phosphorylated histone 3 (p-H3) was performed at 4 dpf, and the pH3- positive cells in the liver were quantified ($n = 8$, $P < 0.05$). (C) Embryos were injected with 8 ng con MO or E1 MO. Then an immunohistochemistry assay involving staining with cell apoptosis marker TUNEL in the liver ($n = 6$) was performed at 4 dpf.

To identify the proper morpholino concentrations for injection, different doses of morpholinos were microinjected into 1- to 4-cell embryos respectively, and embryos were morphologically normal at 2, 3 and 4 dpf when injected with 2, 4, or 8 ng morpholinos, but larger amounts of morpholino (16 ng/embryo) caused abnormal development of embryos (Fig. S4). Morpholino doses of 4 and 8 ng/embryo were used for subsequent experiments. To investigate the developmental functions of HPS in zebrafish, morpholino-mediated gene knockdown was performed in *Tg(fabp10: RFP)* transgenic zebrafish. In this transgenic line, liver-specific expression of RFP (red fluorescence) was clearly visible at 60 hpf. Injection of HPS E1 MO caused no apparent morphological change. However, an obvious reduction in liver RFP expression intensity and region at 3–4 dpf were observed when the HPS E1 MO was injected into 1- to 4-cell embryos (Fig. 2B, C. $P < 0.05$), indicating that knockdown HPS with E1 MO causes the small liver phenotype. In order to confirm this, a morphant-rescue study was performed by microinjecting HPS mRNA into 1- to 4-cell embryos along with morpholinos. As shown in Fig. 2D, E, the HPS MO-induced phenotype was partially rescued by co-injection mRNA encoding zebrafish HPS ($P < 0.05$), supporting the conclusion that the *HPS* MO phenotype is not caused by off-target effects. Taken together, these results showed that HPS has an important role in liver development of zebrafish.

3.3. HPS depletion regulates liver outgrowth in zebrafish

To determine which stage of liver development is affected by HPS depletion, WISH was performed to analyze the expression of the hepatoblast/hepatocyte marker *hhex* in HPS MO- and con MO-injected embryos. At 26 hpf, embryos injected with HPS E1 MO showed normal expression of the *hhex* in the liver primordial region (Fig. 3A). The expression of early endoderm marker *gata6* was analyzed. As shown in Fig. 3B, the expression of *gata6* was identical in MO1 and control embryos at 26 hpf. This suggested that liver specification and budding did not depend on *HPS*. Liver size was assessed by WISH using the specific hepatocyte marker, *fatty acid binding protein 10* (*fabp10*), and results showed that the liver was much smaller than in the control group from 55 hpf to 4 dpf (Fig. 3C). Reductions in liver size can be rescued with HPS mRNA co-injection with the MO (Fig. 3D). These conclusions confirm the results found in Tg transgenic zebrafish. In addition, pancreatic expression of endocrine cell marker *insulin (ins)* was found to remain normal in HPS E1 MO (Fig. 3E), suggesting that pancreatic development was unaffected by HPS depletion.

3.4. Knockdown of HPS causes defective liver cell proliferation

To investigate the mechanism underlying liver size reduction in HPS MOs, immunostaining analysis for cell proliferation and apoptosis were performed. Immunostaining analysis of phosphohistone H3 (pH3), a cell proliferation marker, showed that the hepatocyte proliferation rate in the livers of 4 dpf embryos decreased by more than 75% when microinjected with 8 ng HPS E1 MO compared to 8 ng con MO (Fig. 4A, B. $P < 0.05$). Cell apoptosis by TUNEL cell death assay revealed that embryonic hepatocytes presented a low apoptotic index at 4 dpf, which was unchanged in HPS MO (Fig. 4C). These results indicated that reduction of liver size by HPS MO does not contribute to liver cell death and appears to be due to defective cell proliferation in the developing liver.

4. Conclusion

HPS acts in the regulation of liver regeneration and specific stimulation of hepatocyte proliferation, as described in previous

MO, 8 ng E1 MO, or a combination of 8 ng E1 MO with 200 pg HPS mRNA. Then the WISH assay was performed to detect the mRNA expression of *fabp10* at 55 hpf. Red arrows indicate liver. (E) Embryos were injected with 8 ng con MO or E1 MO. Then WISH assay was performed to detect the mRNA expression of *insulin* at 55 hpf. Red arrows indicate the pancreas.

studies [1,2]. However, its role in vertebrate embryonic development has not been examined previously. In this study, zebrafish were used to investigate the role of HPS in liver development. HPS expression was found to be enriched in the liver throughout hepatogenesis during zebrafish development. *HPS* depletion inhibited liver development and caused small livers in zebrafish. Further analysis suggested that the HPS MO phenotype was attributable to a reduction of hepatocyte proliferation without affecting apoptosis. HPS mRNA injection rescued the small liver phenotype caused by HPS MO-injection. Taken together, these results indicate that *HPS* is important for the later development of zebrafish liver.

Previous studies have shown that hepatotrophic growth factors have an important role in zebrafish liver development. For example, the fibroblast growth factor (FGF) signaling pathway regulates liver homeostasis in zebrafish, and augmenter of liver regeneration (ALR) promotes liver growth during zebrafish hepatogenesis [17,18]. Importantly, the structure of HPS is similar to angiopoietin-like factors (Angptls). Recently, angptl3 was reported to be primarily expressed in developing zebrafish livers and Angptl3 depletion could reduce the size of the developing liver. Preliminary analysis showed that the HPS was specifically expressed in normal mouse liver cells and highly up-regulated in liver regeneration [8,19]. The present study showed that HPS was initially expressed at 24 hpf and continued to be highly expressed in the developing livers. Knockdown of HPS resulted in an obvious reduction in liver size. Further studies revealed that HPS promotes liver growth by stimulating hepatocyte proliferation rather than inhibiting apoptosis or reducing hepatocyte cell size. In this way, the current result demonstrates that HPS should be placed on the growing list of genes known to regulate vertebrate hepatogenesis.

A small liver phenotype may be caused by the failure of the liver bud or proper differentiation. Knockdown of HPS did not affect the expression of early liver or of the pancreas development marker *hhex*. The expression of early endoderm marker *gata6* and later pancreas marker *insulin* were detected and found to be almost the same between HPS MO1- and control-injected embryos. These results indicated that HPS knockdown-induced liver size reduction is not caused or partially caused by interfering with early hepatic specification.

Recently, a study suggested a previously unrecognized role for HPS in regulation of intermediate metabolism [8,9]. HPS knockout mice showed an overall glucose and lipid metabolism disorder not observed in wild type mice. This article announced that HPS was also expressed in adipose tissue and may be involved in the regulation of adipose tissue morphology and function [8]. Future investigations are required to elucidate the role of HPS in metabolism of zebrafish.

In conclusion, this is the first study to indicate a crucial role of HPS in vertebrate hepatogenesis. HPS knockdown causes specific defects in hepatocyte proliferation but not in liver initiation. The knowledge gained from this study shows the regeneration function of HPS and confirms the application of HPS on liver transplantation and tissue engineering.

Conflict of interest

The authors declared that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.121.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.121.

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