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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