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Zebrafish heparin-binding neurotrophic factor enhances neurite outgrowth during its development

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Abstract

Heparin-binding neurotrophic factor (HBNF) is a secreted heparin-binding protein containing highly basic and cysteine-rich amino acid residues. In this study, we cloned the full-length *HBNF* cDNA from zebrafish and determined its genomic structure by bioinformatics analysis. Zebrafish *HBNF* gene is composed of five exons and four introns spanning approximately 82 kb. RT-PCR analysis revealed that zebrafish *HBNF* transcript was highly expressed in adult brain and intestine tissues while less in other tissues. During embryogenesis, zebrafish *HBNF* transcript was observed to be moderately expressed at earlier stages with a gradual decline. Higher expression level was observed after hatching and maintaining this level into adulthood. The overall amino acid sequence of zebrafish HBNF shows 60% identity to human HBNF, but with approximately 40% identity to other midkine proteins. Like mammalian homolog, zebrafish HBNF could induce significant neurite outgrowth in PC12 cells without NGF stimulation. In addition, zebrafish HBNF was able to enhance extensive neurite outgrowth in zebrafish embryos during embryogenesis. In summary, a feasible in vivo assay for neurite outgrowth was established in zebrafish.
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Keywords: Heparin-binding neurotrophic factor; Neurite outgrowth; PC12 cells; Zebrafish; Neuron-specific promoter

Heparin-binding neurotrophic factor or neurite-promoting factor (HBNF) was first copurified with bovine acidic fibroblast growth factor from brain tissues [1]. It is a secretory heparin-binding protein with highly basic and cysteine-rich amino acid residues. In mammals, HBNF shares 50% identity with midkine (MK) and they constitute a new family of heparin-binding proteins [2]. HBNF and MK are not only functionally related proteins with similar promotion of neurite extension in PC12 cells [3,4], but also structurally related proteins. They have 10 conserved cysteine residues, a highly conserved hinge region as well as two clusters of basic residues for heparin binding [5]. The genomic structures of human *HBNF* gene [6] and *MK* gene [7] are also similar, comprising of five exons and four introns with similar exon/intron junction. Recently, *HBNF* and *MK* genes are proposed to evolve from gene duplication before divergence of tetrapods and fish [5]. In addition to neurite outgrowth promotion, HBNF also has a variety of biological activities, such as stimulating cell growth, acting as an angiogenesis factor, and containing oncogenic

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activity [8]. Therefore, it is also known as pleiotrophin (PTN) [9] and heparin-binding growth-associated molecule (HB-GAM) [10].

It has been proposed that HBNF/PTN is evolutionally conserved according to the published sequences from various species [11]. However, the complete zebrafish *HBNF* transcript has not been reported. In this study, we cloned the full-length cDNA of zebrafish *HBNF* mRNA and determined its genomic structure. The biological activity of zebrafish HBNF was assayed in PC12 cells as well as in zebrafish embryos. We found that zebrafish HBNF shared 60% identity with human HBNF and only 40% identity with human MK, zebrafish mdka, and mdkb. In addition, zebrafish HBNF/PTN could promote significant neurite outgrowth in vitro and in vivo.

Materials and methods

Materials. All restriction enzymes were purchased from Promega Biosciences, (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). Chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (MO, USA).

Cell cultures. Rat pheochromocytoma PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, HyClone, Utah), penicillin G (50 U/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM) in a humidified atmosphere of 5% CO_2 at 37 °C. Various culture reagents used were purchased from HyClone (Logan, Utah).

Fish. Zebrafish (*Danio rerio*) were maintained at 28 °C on a 14-h light/10-h dark cycle. Embryos were incubated at 28 °C and different developmental stages were determined according to the description in Zebrafish Book [12].

Total RNA isolation and first-strand cDNA synthesis. Total RNA was isolated from the fertilized eggs, different embryonic stages (0, 5, 12, 24, 48, and 72 h postfertilization), and from various tissues (brain, gill, intestine, muscle, and ovary) of adult zebrafish using the RNAzol reagent (Tel-Test) according to the instructions of the manufacturer. After treatment with RQ1 RNase-Free DNaseI (Promega), 50–100 μg of total RNA from each tissue was used for the first-strand cDNA synthesis in a 25 μl reaction mixture containing 10 pmol of oligo(dT) primer and 100 ng of random primer (Promega), 30 U RNasin (Promega), 1 mM dNTP, 10 mM dithiothreitol, and 300 U of Superscript II RT (Invitrogen Life Technologies, CA). The reaction mixture was incubated at 42 °C for 1 h. Two microliters of the cDNA products was used for subsequent PCR amplification.

Isolation of the full-length HBNF cDNA from zebrafish. In order to isolate the cDNA covering the complete open reading frame (ORF) of zebrafish *HBNF*, according to the sequences of one zebrafish EST (Accession No. AL925602), the 3′-end of zebrafish *HBNF* mRNA was obtained by the 3′-RACE PCR technique. For 3′-RACE, cDNA was amplified with a zebrafish *HBNF* sense primer, zHBNF-F1: 5′-CAA ACT ATT CCT CAG ACT ACA GCA TGC-3′, and an oligo(dT) primer. The PCR product was reamplified with a nested zebrafish *HBNF* sense primer, zHBNF-F2: 5′-GCA TGC AGC AGC AGT GGG TGT GTG TG-3′ and an oligo(dT) primer. PCR amplification was performed in a 50 μl reaction mixture containing 2 μl first-strand cDNA, 0.5 μg primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U ExTaq (Takara Shuzo, Shiga, Japan).

The samples were incubated in a thermal cycler (Hybaid Multi-Block System, Hybaid Limited, MA). The cDNA from brain as template was used for PCR amplification using the program of 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s; and the final extension at 72 °C for 15 min. All RACE products were ligated into pGEM-T easy vector (Promega) and subjected to sequence analysis.

RT-PCR analysis of zebrafish HBNF mRNA. PCR amplification was performed in a 50 μl reaction mixture containing 200 ng zHBNF primers (zHBNF-F3, 5′-ATC GGA CTG TGG AGA GTG GCA GTG GAG TGT G-3′ and zHBNF-R1, 5′-TGG TCT TGG GTT TGC CAC AGG GTT TGG TGG C-3′) or β-actin primers (ActF, 5′-CCT CCG GTC GTA CCA CTG GTA T-3′ and ActR, 5′-CAA CGG AAG GTC TCA TTG CCG ATC GTG-3′), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U ExTaq (Takara Shuzo, Shiga, Japan). The samples were incubated in a thermal cycler (Hybaid MultiBlock System) at 96 °C for 3 min; 40–50 cycles of 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and the final extension at 72 °C for 5 min. A negative control was performed in the absence of first-strand cDNA. All PCR products were separated on a 1.2% agarose gel.

DNA sequence analysis. DNA sequence analysis was performed by using PRISM Ready Reaction Big-Dye Termination Cycle sequencing Kit (Applied Biosystems, CA) on an Applied Biosystems 310 automated DNA sequencer. Sequence analysis was performed by using the Clustal X and GenDoc programs.

Construction of expression plasmid and cell transfection. The expression plasmid, pcDNA-HBNF-HA, was constructed by inserting the full-length *zHBNF* cDNA into pcDNA3-HA at the *Bam*HI and *Eco*RI sites, which allows the generation of the HBNF protein with in-framed HA tag at the C-terminal. The pcDNA3-HA plasmid was derived from pcDNA3 plasmid by adding the influenza A virus hemagglutinin (HA) tag (YPYDVPDYA) into pcDNA3 at the *Eco*RI and *Xho*I sites. The pHuC-GFP plasmid contains the GFP gene as the reporter gene and is driven by a zebrafish neuron-specific HuC promoter [13]. To investigate whether the zebrafish HBNF could induce neurite outgrowth in vitro, the full-length *HBNF* cDNA under the control of CMV promoter was transiently transfected into PC12 cells. PC12 cells at 80% confluence were grown in a six-well dish and transiently transfected for 6 h at 37 °C with 1 μg pcDNA-HBNF-HA by using the Lipofectamin Kit (Life Technologies, MA). After 48 or 72 h cells were observed under an Olympus IX70-FLA inverted fluorescence microscope. Images were taken by using the SPOT system (Diagnostic Instruments, Sterling Heights, MI) and assembled by PhotoShop program (Adobe System, CA). In addition, serum-free supernatants of PC12 cells transfected with pcDNA-HBNF-HA were collected at 72 h after transfection.

Western blot analysis. Monoclonal antibody against HA tag was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblot analyses were performed by separating the supernatants of PC12 cells transfected with pcDNA-HBNF-HA on 10% SDS–PAGE, followed by transferring to a PVDF membrane (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 4% skim milk in PBS and then incubated with anti-HA monoclonal antibody (1:3000 dilution) or anti-GFP polyclonal antibody (1:3000 dilution) at 4 °C, overnight. After washing with PBST (0.2% Tween 20 in PBS) for three times, the membranes were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (1:5000, Amersham Biosciences, Piscataway, NJ) at room temperature for 2 h. The membranes were washed as described as above, and signals were detected using enhanced chemiluminescence (ECL) (NEN Life Science Products, Boston, MA).

Microinjection of expression plasmid into zebrafish embryos. The expression constructs, pcDNA-HBNF-HA, and pHuC-GFP, were linearized by digestion with restriction enzyme *Sca*I, purified with PCR cleanup/Gel extraction kit (Qiagen GmbH, Germany). DNA concentration was adjusted to 100 μg/ml in 0.1 M KCl solution containing 0.5% phenol red and 100–200 pl of pHuC-GFP alone or in combination with pcDNA-HBNF-HA was microinjected into the zebrafish embryo at one-cell stage by using Narishige IM 300 microinjector system (Narishigie Scientific Instrument Laboratory, Tokyo, Japan). Embryos

at 48 and 72 h postfertilization were observed under an Olympus IX70-FLA inverted fluorescence microscope. Images were taken by using the SPOT system and assembled by PhotoShop program.

Results

Isolation of zebrafish HBNF cDNA

To study the function of zebrafish HBNF, we started with isolating the *HBNF* cDNA. We used the coding region of the human HBNF (Accession No. M57399) to search the GenBank database for related expression sequence tag (EST) sequences by using the program tBLAST. One corresponding EST clone (Accession No. AL925602) was found in zebrafish, which contained 97 bp of the 5′-untranslated regions (UTR), translation initiation codon ATG, and incomplete coding region. Therefore, we used 3′-RACE to obtain the other coding region and 3′-UTR. The assembled full-length *HBNF* cDNA consists of 994 bp containing an open reading frame of 477 bp encoding a protein of 158 amino acid residues. The *HBNF* cDNA sequence was deposited in GenBank with an Accession No. of AY572239. The overall amino acid sequence comparison indicates that zebrafish HBNF displays 60% identity to human HBNF, while only 39–40% identity to human midkine, zebrafish mdka, and mdkb (Fig. 1). However, 10 conserved cysteine residues as well as one highly conserved hinge region are present in all five members of this HBNF/midkine family. Two thrombospondin repeat (TSR) domains for heparin binding are also conserved in all members of this family.

Genomic structure of zebrafish HBNF gene

We have used the 994 bp of *HBNF* cDNA (Accession No. AY572239) as query to search the GenBank non-redundant database using BLAST program. The *HBNF* cDNA matched to five non-contiguous regions in a zebrafish BAC clone CH211-278F21 (GenBank Accession No. BX088688). Subsequently, alignment of the sequence of BAC CH211-278F21 to that of *HBNF* cDNA by BLAST 2 program indicated that *HBNF* cDNA is contained within five putative exons. Using these

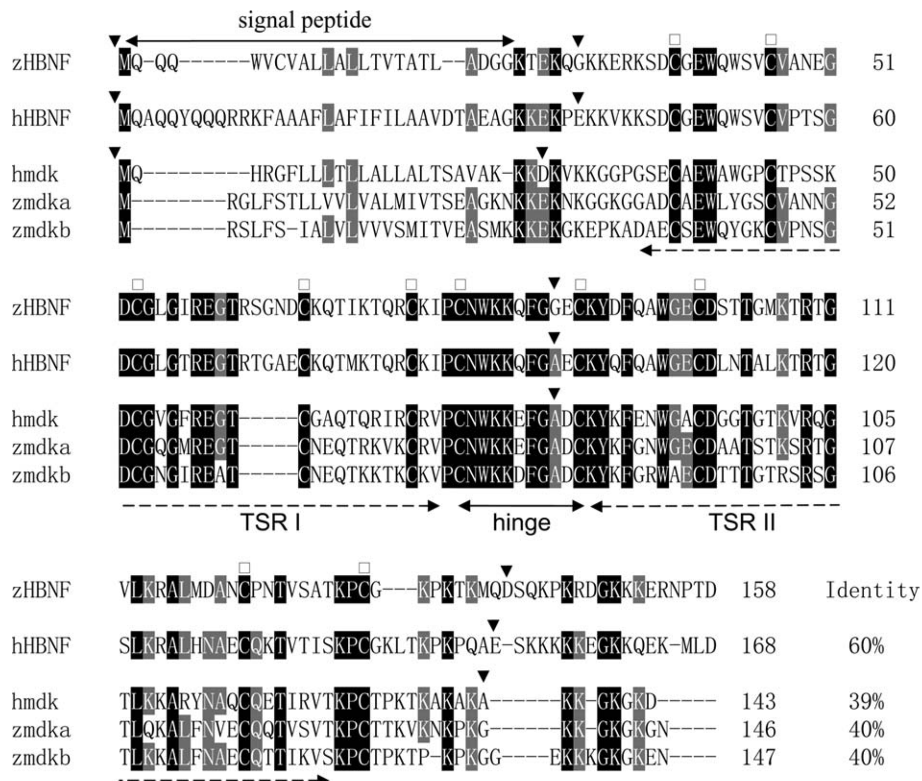

Fig. 1. Alignment of amino acid sequences of zebrafish HBNF with other members of the MK family. Alignment of amino acid sequences of zebrafish HBNF with human HBNF/PTN and other MK proteins. Amino acid sequences of human HBNF/PTN and various midkine proteins from zebrafish and human were aligned using CLUSTAL X program. Gaps are introduced to optimize alignment and shown as dashes. Sequences used are: human HBNF/PTN (NM_002825), human mdk (AAA58478), and zebrafish mdk-a (AF503614) and mdk-b (NM_131716). All five identical amino acids are shown in black boxes while four identical amino acids in grey boxes. Conserved cysteines are indicated by squares. Other regions of putative signal peptide, two thrombospondin repeat (TSR) domains, and one conserved hinge region are also shown. Percentage identity of amino acid sequence between zebrafish HBNF and other members of the MK family is shown. Spliced sites are indicated by down-pointing arrowheads. It is obvious that most of the spliced sites are highly conserved.

putative exons as a model, a sequence alignment was produced such that each intron concurred with the omitted GT/AG intron donor/acceptor site rule [14]. Exon 1 contains 97 bp of 5′-UTR and exon 2 the next 1 bp of the 5′-UTR and the first 88 bp of the coding sequences of the *HBNF* cDNA. Exons 3 and 4 contain the next 174 and 159 bp of the coding sequences while exon 5 contains the last 56 bp of the coding sequences and 419 bp of 3′-UTR. The size of four introns varied considerably, ranging from 1763 bp (intron 2), 5298 bp (intron 3), 14,749 bp (intron 4) to 60,277 bp (intron 1) with an average size of 20,522 bp. The *HBNF* gene spans approximately 83 kb. It is obvious that intron/exon junctions of zebrafish *HBNF* gene, human *HBNF*, and *midkine* genes are similar, suggesting that they may evolve from a common ancestor.

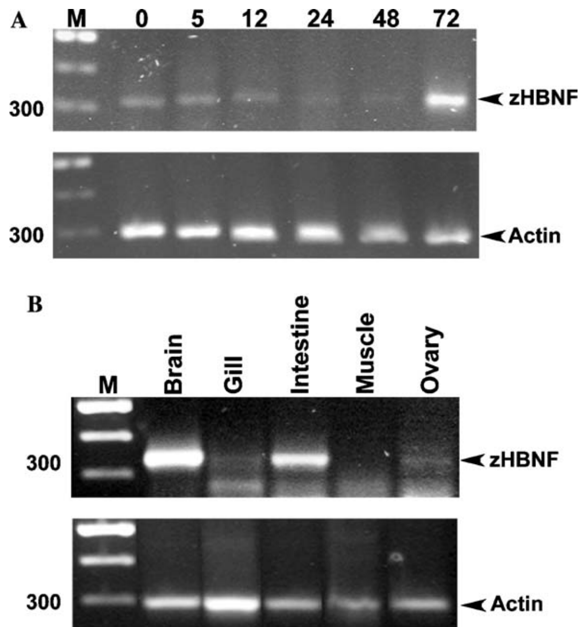

Fig. 2. Expression profile of zebrafish HBNF mRNA in embryos from different developmental stages and various adult tissues. Total RNA (5–10 μg) isolated from different embryonic stages (A) and various adult tissues (B) was subjected to RT-PCR analysis. The resulting PCR products were separated on a 1.2% agarose gel. The intensity of 300 bp DNA fragment using β-actin-specific primers amplified from zebrafish tissues was used to evaluate the relative amount of cDNA used in each PCR.

Expression profiles of zebrafish HBNF transcripts by RT-PCR analysis

In order to determine the expression pattern of *HBNF* mRNA, zebrafish embryos at different developmental stages and various tissues from adult zebrafish were collected for cDNA preparation. The amount of *β-actin* transcript was used as internal control for normalization. Our data showed that the *HBNF* mRNA was readily detected in embryos upon fertilization (0 h), and its level remained constant for at least 12 h after fertilization and declined gradually. At 72 h postfertilization, the expression of *HBNF* transcript was higher than that at any stage during development (Fig. 2A). It was in agreement with earlier report that the expression of human *HBNF* mRNA was observed to gradually increase during embryogenesis, reaching a maximal level at birth and maintaining this level into adulthood [3]. In adult fish, *HBNF* is highly expressed in brain and intestine which is consistent with the biological function of HBNF. Moderate expression of *HBNF* mRNA was found in ovary, less in gill and muscle.

Expression of zebrafish HBNF cDNA in PC12 cells

To test whether the zebrafish HBNF could induce neurite outgrowth in vitro, the full-length *HBNF* cDNA under the control of CMV promoter was transiently transfected into PC12 cells. As shown in Fig. 3, the control cells were round without differentiation (panel A), while the cells transfected with pcDNA-HBNF-HA

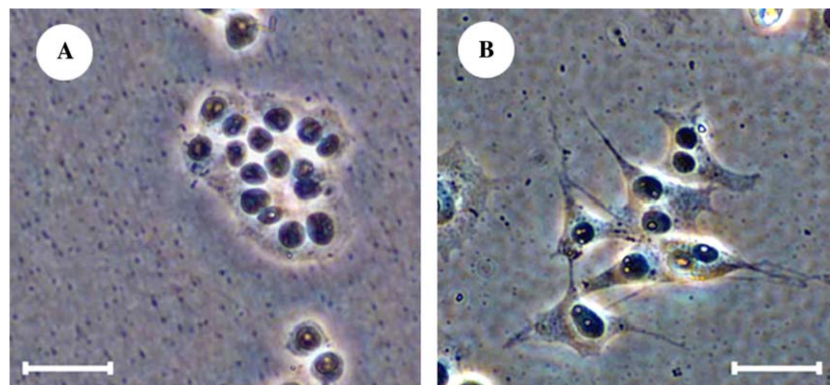

Fig. 3. Zebrafish HBNF induces neurite outgrowth in PC12 cells. PC12 cells were transfected with one microgram of pcDNA3-HA (A) or pcDNA-HBNF-HA (B). After 72 h posttransfection, cells were observed under inverted microscope. Images of cell morphology were taken by using the SPOT system. Bars, 10 μm.

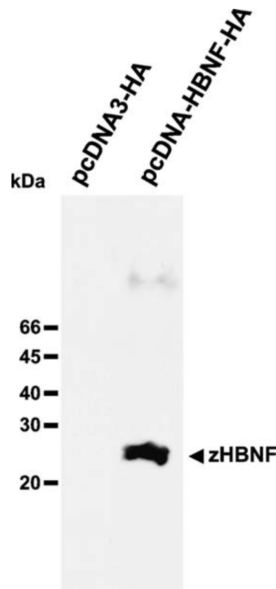

Fig. 4. Secretion of zebrafish HBNF in the culture medium of PC12 cells. PC12 cells were transfected with one microgram of pcDNA3-HA or pcDNA-HBNF-HA. After 72 h posttransfection, serum-free supernatants of transfected PC12 cells were collected and subjected to Western blot analysis by using anti-HA monoclonal antibody.

underwent differentiation with some long neuritis extended from these cells at 72 h after transfection (panel B). On the other hand, a secreted protein was detected in the supernatant of these cells transfected with pcDNA-HBNF-HA (Fig. 4). These data indicated that transient expression of zebrafish HBNF could induce weak but significant neurite outgrowth in PC12 cells.

HBNF enhances neurite outgrowth in zebrafish embryos

To further investigate the biological activity of zebrafish HBNF in vivo, a neurite outgrowth promotion assay using zebrafish embryos, in a mosaic fashion, was developed to study the enhancement of neurite outgrowth extended from GFP-labeled neurons during zebrafish development [15]. In this study, we used the *GFP* gene as the reporter gene driven by a neuron-specific promoter, *HuC* promoter [13]. The expression construct of pcDNA-HBNF-HA was coinjected with pHuC-GFP into zebrafish embryos at one-cell stage. Several expression patterns with strong enhancement of neurite outgrowth in this assay are shown in Fig. 5. It is interesting to note that pHuC-GFP alone displayed stronger GFP expression in trigerminal ganglion, axon as well as motor neurons (panel 1). However, coinjection of pcDNA-HBNF-HA and pHuC-GFP not only showed similar GFP expression pattern as that of pHuC-GFP alone, but also displayed significant enhancement of neurite outgrowth (panels 2, 3, and 4). Other zebrafishes with neurite outgrowth enhancement were also presented, but with different branching patterns. Some neurites covering the yolk ball were extended from one neuron (panels 5 and 8), while other neurites extended to reach the margin of dorsal and ventral fins (panels 6, 7, 9, and 10). These data indicate that the expression of zebrafish *HBNF* could induce extensive neurite outgrowth with wider and complicated branching patterns in zebrafish embryo.

Discussion

In this study, we cloned the full-length cDNA of zebrafish *HBNF* gene and determined its genomic structure. Like human pleiotrophin (PTN) gene [6], zebrafish *HBNF* gene is composed of five exons and four introns and spans approximately 82 kb, compared to approximately 115 kb for human gene. The intron 1 and intron 4 of both genes are large, 60.3 and 14.7 kb, respectively, for zebrafish *HBNF* gene, while 88.5 and 23.2 kb for human *PTN* gene. Interestingly, another member of midkine family, *MK* gene, has similar genomic structure containing five exons and four introns, but only spans 4 kb [7]. Due to conservation of intron/exon junction, these two genes are proposed to arise by gene duplication during the evolution. Recently, a fish-specific duplication of midkine (*MK*) gene was identified and two *mdka* and *mdkb* genes were found in zebrafish [5].

The structure of human PTN and its interaction with heparin have been determined [8]. HBNF/PTN contains two β-sheet domains that are similar to the thrombospondin type I repeat (TSR) found in MK protein [16,17] and other extracellular proteins [18]. PTN has high affinity binding to heparin and the binding sites are identified within the β-sheet domains. As shown in Fig. 1, zebrafish HBNF also contains two similar TSR domains separated by a highly conserved hinge region, with 80% and 70% identity, respectively, to those TSR domains found in human PTN. The C-terminal lysine-rich region of HBNF/PTN is longer than that of MK proteins.

Initially, the function of HBNF/PTN was found to promote neurite outgrowth from different cultured neuronal cell types, including primary embryonic cortical neurons [19,20], PC12 cells [1,21], and neuroblastoma cells [22]. PC12 cells are derived from a transplantable rat pheochromocytoma with an important feature of responding to nerve growth factor (NGF) to differentiate into neuron-like cells. Upon exposure to NGF, PC12 cells cease proliferation, and extend neuritis [23]. Without NGF, zebrafish HBNF could induce weak but significant neurite extension in PC12 cells, when these cells were transfected with zebrafish HBNF cDNA (Fig. 3). These data suggested that the expression of zebrafish *HBNF* is not sufficient to achieve extensive neurite outgrowth in PC12 cells.

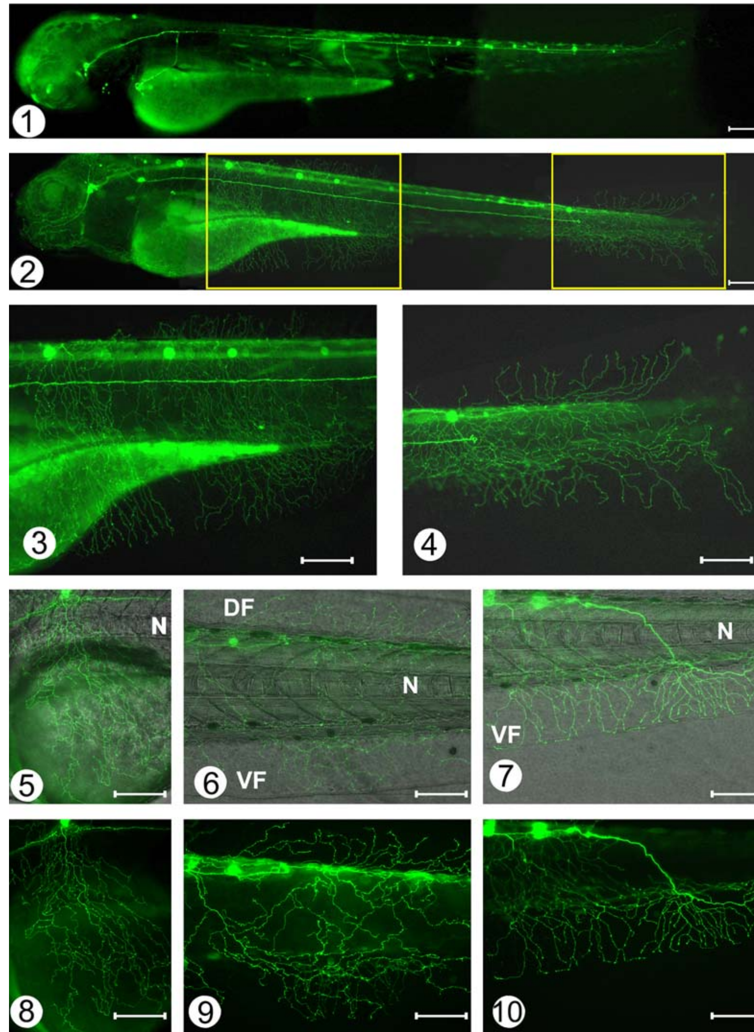

Fig. 5. Enhancement of neurite outgrowth by HBNF protein in zebrafish embryos. The expression constructs pcDNA-HBNF-HA and pHuC-GFP (panels 2, 5–7) were coinjected into zebrafish embryos at one-cell stage. Similar injection of pHuC-GFP alone (panel 1) was used as control. Zebrafish embryos at 72 h postfertilization with GFP fluorescence were selected for image analysis. Embryos are shown as lateral view with anterior to the left. Experiments were repeated at least three times and at least 100 fertilized eggs were used for injection of expression constructs at each time. Four representative patterns with significant enhancement of neurite outgrowth (panels 2, 5–7) are shown. Images of bright field and fluorescence were merged and are shown in panels 1, 2, and 5–7, while fluorescence images only in panels 3, 4, and 8–10. Higher magnification of two regions marked with yellow boxes in panel 2 is shown in panels 3 and 4, respectively. N, notochord; DF, dorsal fin; and VF, ventral fin. Scale bars: 100 μm (1, 2, 3, and 4); 50 μm (5 and 8); and 33 μm (6, 7, 9, and 10).

So far, there is no suitable in vivo assay to assess the enhancement of neurite outgrowth by neurite-promoting factors. In this study, we used zebrafish embryos to establish an in vivo system that can be used to study the enhancement of neurite outgrowth from GFP-labeled live neurons during zebrafish development [15]. Zebrafish embryos could provide more additional factors than those in PC12 cells for extensive neurite outgrowth. In zebrafish, *HuC* gene has been shown to be a useful early marker for neurons and a 2.8 kb promoter region of this gene is sufficient to direct *GFP* expression in a neuron-specific pattern closely similar to endogenous *HuC* expression [13]. As shown in Fig. 5, pHuC-GFP alone displayed *GFP* expression in trigerminal ganglion, axon as well as rohon beard (RB) or motor neurons without visible branched and long neurites (panel 1). However, coinjection of pcDNA-HBNF-HA and pHuC-GFP resulted in significant enhancement of neurite outgrowth with extensive branched and long dendrites (panels 2, 3, and 4). Other injected zebrafishes have different dendrite branching patterns, but with similar robust neurite outgrowth. For example, many neurites descend and cover the yolk ball (panels 5 and 8) while both ascending and descending neurites reach the margin of dorsal and ventral fins (panels 6, 7, 9, and 10). For the first time, these data indicated that the expression of zebrafish *HBNF* could induce robust neurite outgrowth with wider and complicate branches from GFP-labeled neurons during zebrafish development.

HBNF/PTN was first isolated as a heparin-binding protein that was eluted from a heparin affinity column with high salt concentration [1]. Such a high affinity-binding property suggests that heparin or heparin-type carbohydrates may play important roles in the biological function of HBNF/PTN. Indeed, further studies demonstrate that a transmembrane heparan sulfate proteoglycan, *N*-syndecan (syndecan-3), acts as a receptor for HBNF/PTN [24]. Both the heparan sulfate side chains of *N*-syndecan and polyclonal anti-*N*-syndecan inhibit HBNF/PTN-induced neurite outgrowth in the cultured neurons. In addition to *N*-syndecan heparan sulfate, the low molecular weight heparin displays more potent inhibition of HBNF/PTN-induced neurite outgrowth [25,26]. On the other hand, in this study, we established an in vivo neurite outgrowth assay in zebrafish embryos that provided a direct observation of HBNF-induced neurite outgrowth from GFP-labeled neurons during zebrafish development. The inhibitory effect of heparin on HBNF-induced neurite outgrowth in this assay was performed by further injection of different dosage of heparin into zebrafish embryo at two- or four-cell stage. In agreement with previous report [25], heparin could inhibit HBNF-induced neurite outgrowth in zebrafish embryos (data not shown). Further work is needed to compare the inhibitory effect of HBNF/PTN-induced neurite outgrowth in vivo by heparin and its modified forms, *N*-syndecan-derived saccharides, and other glycosaminoglycans.

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