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Alternative splicing transcription of *Megalobrama amblycephala* HIF prolyl hydroxylase *PHD3* and up-regulation of *PHD3* by HIF-1α

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ABSTRACT

PHD3 is a hydroxylase that hydroxylates prolyl residues on hypoxia-inducible factors (HIFs) in mammals. In this study, the full-length cDNA and promoter sequences of *Megalobrama amblycephala PHD3* gene were isolated by a modified RACE method. *PHD3* cDNA was 1622 bp in length, including an ORF of 717 bp encoding 238 amino acid residues. The semi-quantitative PCR results suggested that *PHD3* was highly expressed in liver in the normal condition, while after hypoxia treatment this gene was significantly increased in all analyzed tissues. *PHD3* was detected only in the initial stages of *M. amblycephala* embryo development. In addition, the presence of another alternatively processed *PHD3* transcript, designated *PHD3Δ1* was observed in the process of analyzing the expression of *PHD3*. Both *PHD3* and *PHD3Δ1* were up-regulated under hypoxia, and had five the hypoxia response elements (HREs) by *in silico* scanning on the promoter. Further luciferase assay indicated that all HREs significantly responded to hypoxia. Taken together, these results suggest that *PHD3* plays important roles in hypoxia response and early embryo development of *M. amblycephala*.

1. Introduction

Hypoxia-inducible factors (HIFs), the key hypoxia response factors, that are robustly induced under hypoxia to increase hundreds of proteins in several biological processes, including angiogenesis, erythropoiesis, cell-cycle arrest, apoptosis and metastasis to response hypoxia stress [1,2]. The heterodimeric HIFs are composed of a labile hypoxia-regulated α subunit and a constitutive β subunit. HIF-αs (including HIF-1α, HIF-2α and HIF-3α) proteins are hydroxylated by prolyl hydroxylases (PHDs) on conserved prolyl residues Pro^{402} and Pro^{564} in the conserved LXXLAP motif in human [3–5]. The hydroxylated HIF-αs are recognized by the von Hippel-Lindau tumor suppressor protein (pVHL), and finally degraded by 26S proteasome under normoxia conditions [6]. PHDs require molecular oxygen to activate catalytic activity, therefore prolyl hydroxylation does not occur under hypoxia conditions, leading to instantaneous stabilization and accumulation of HIF-α proteins [7–9]. HIF-αs then migrate to the nucleus, dimerize with HIF-β and recruit the transcriptional coactivator p300, and they both bind to HREs comprising a core 5′-[A/G]CGTG-3′ consensus sequence and highly variable flanking sequences on the promoters of HIF-responsive genes [10].

The three human HIF-α prolyl hydroxylases that have been characterized so far (PHD1-PHD3) belong to the family of 2-oxoglutarate-dependent, non-haem iron-binding dioxygenases [7,8,11]. PHD2 and PHD3 are reported to be hypoxia-inducible [12,13], and PHD2 protein possesses a MYND (myeloid, Nervy, and DEAF-1) type zinc finger interaction domain that could distinguish it from PHD1 and PHD3 [14]. The MYND domain is reported to anchor PHD2 via the FKBP38 protein to the mitochondrial or endoplasmic reticulum membranes to keep cytosolic PHD2 stable [15].

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In human, HIF- deficient or suppressed cells do not up-regulate PHD3 in response to hypoxia [16]. Moreover, dysregulation of HIF-α in pVHL-deficient renal clear cell carcinoma cells is associated with the loss of hypoxia PHD3 induction [17]. In human, PHD3 has been experimentally identified as a HIF-1α direct target [18], suggesting that PHDs and HIF-α form a feedback loop that limits hypoxia signaling and accelerates HIF-α degradation after reoxygenation. PHD3 induces apoptosis in rat cells, while in breast, cervical, prostate and colorectal cancer cell lines, the DNA methylation status on the PHD3 promoter is enhanced [19–23], as a result, PHD3 expression is suppressed, leading to uncontrolled cancer cell growth. The fact that PHD3 is silenced in such a wide variety of tumor types suggests that PHD3 affects important signaling pathways that are common to the progression of many malignancies [24–26].

Compared to mammals, the information regarding fish *PHD3* is extremely limited. The experimentally identified *PHD3* mRNA sequence of fish is only available in *Danio rerio* (zebrafish) and *Cyprinodon variegatus*. Since aquatic environments exhibit much wider temporal and spatial variations of oxygen concentrations, leads fish frequently exposed to hypoxia conditions. *PHD3* as a regulator for HIF-1α, its role in fish hypoxia adaption is unclear. In the present study, we identified and characterized *Megalobrama amblycephala PHD3* gene and promoter, and examined the spatio-temporal expression patterns. In addition, the *PHD3* isoforms and regulation by HIF-1α were also studied.

2. Materials and methods

2.1. Experimental fish

M. amblycephala (mean weight 50 g) were obtained from Tuanfeng breeding base in Hubei Province, China. Fish were acclimated for two weeks with daily diet and controlled photoperiod (14 h/day and 10 h/night) in one 500 L tank with circulating water system, 5.5 mg/L of dissolved oxygen (DO) and 25 °C of temperature in water were maintained. To analyze the temporal expression of *PHD3*, fertilized eggs and larval of *M. amblycephala* at 0, 1.08, 2.83, 4.42, 5.17, 10.6, 12.3, 15.7, 17.5, 23.9, 32.0, 39.4, 48.4, 72.2, 94.4, 144, 240 and 288 h post-fertilization (hpf) were collected. All eggs and larval were cultured in the normoxia conditions as mentioned above.

An automatic hypoxia control machine was used in this experiment to maintain DO by increasing or decreasing the nitrogen gas flowed into the 100 L hermetic tank. Ten fish were randomly divided into two groups: the control (DO: 5.5 ± 0.2 mg/L, T: 25 °C) and hypoxia group (DO: 1.0 ± 0.2 mg/L, T: 25 °C). When the DO level reached the stated values in less than 30 min, fish were quickly anesthetized with MS-222 (150 mg/L), and various tissues including blood (Bl), liver (L), spleen (S), muscle (M), brain (Br), gill (G), heart (H), intestine (I) and kidney (K) were immediately excised and subsequently frozen in liquid nitrogen for RNA isolation. Meanwhile, genomic DNA was extracted from fish fin clip using the traditional phenol-chloroform method.

2.2. Isolation of full-length cDNA and promoter sequences of PHD3

The full-length cDNA and promoter sequences of *M. amblycephala PHD3* were cloned using a modified full-length cDNA amplification strategy based on bioinformatics technology and multiplexed PCR methods as previously described [27]. Briefly, the core sequence of *PHD3* gene was isolated by tBLASTn program from already published *M. amblycephala* transcriptome [28], and then the core sequence was validated by PCR using PHD3-core primers. The total RNA extracted from *M. amblycephala* was reversed by hairpin structure oligo(dT)-anchor primer to obtain first-strand cDNA library. The cDNA library was used as template to amplify the 3′ end of *PHD3* gene by 3′-F (designed according to core sequence), Outer-R and Inner-R primers (designed according to the anchor sequence). After 3′ end and core sequences assembling, sequence-specific reverse primers (SPR1, SPR2 and SPR3, primers were designed based on the assembled sequence) were synthesized and together with arbitrary degenerate primers (AD1, AD2 and AD3) to amplify the 5′ sequence of *PHD3* gene by TAIL-PCR in cDNAs. The sequences were re-assembled and aligned to zebrafish genome database to analyze exon number and distribution. The PCRs in cDNAs will continue unless the 5′ sequence of multi-assembled sequence reached zebrafish *PHD3* exon1 region after aligning, and then another TAIL-PCR using genomic DNA as template was performed to obtain the remaining *M. amblycephala PHD3* gene 5′ and promoter sequences. The finally assembled sequence is conducted *in silico* analysis to predict the transcription start site (TSS). The putative 5′ end of *PHD3* gene was further validated by primers corresponding to these predicted sites in cDNAs.

2.3. Sequence analysis

The proximal promoter sequence of the *PHD3* gene was uploaded to online JASPAR CORE Vertebrata database (http://jaspar.genereg.net/) to scan the common *cis*-acting elements. The EMBOSS Cpgplot (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot) was used to predict the CpG island on the *PHD3* promoter and exon1 regions. The amino acid sequence of PHD3 was predicted using a translator program at open reading frame (ORF) finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and protein parameters were predicted by ProtParam tool (http://web.expasy.org/protparam). While, the protein domains were marked according to UniProt (http://www.uniprot.org/) and SMART (http://smart.embl-heidelberg.de/).

2.4. Semi-quantitative assay

The expressions of *M. amblycephala PHD3* in different tissues and developmental stages in normoxia and hypoxia conditions were analyzed. According to the previously reported studies in *M. amblycephala*, *ACTB* was selected as the internal gene [29]. Semi-quantitative PCR reaction was performed as follows: incubation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 20 s, 60 °C 20 s and 72 °C for 10 s. 20 ng of each tissues' cDNA was used as template in a total of 20 μL reaction mixture, and after amplification equal volume of 5 μL PCR products for every samples were quantified by agarose gel electrophoresis.

2.5. Construction of recombinant plasmid

The *in silico* analysis indicated that *PHD3* gene had HREs on the promoter. Thus, the promoter sequences of *PHD3* and *VEGF* (as a positive control) were amplified and subcloned into the pGL-3basic vector (Promega, USA) between *Xho* I/*Hind* III sites to identify active HRE in response to HIF-1α. All primers used in this study were presented in Table 1.

2.6. Cell culture and treatment

HeLa cells were cultured in DMEM:F12 supplemented with 10% fetal bovine serum (Hyclone, USA), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Life technologies, USA) in humidified incubators containing 5% CO_2. Cells were grown to ~60% confluency and transfected with recombinant plasmids pGL3b-F1, pGL3b-F2,