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# Identification and characterisation of the anti-oxidative stress properties of the lamprey prohibitin 2 gene

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ABSTRACT

The highly conserved protein prohibitin 2 (PHB2) has been implicated as a cell-surface receptor in the regulation of proliferation, apoptosis, transcription, and mitochondrial protein folding. In the present study, we identified a *Lampetra morii* homologue of PHB2, Lm-PHB2, showing greater than 61.8% sequence identity with its homologues. Phylogenetic analysis indicated that the position of Lm-PHB2 is consistent with lamprey phylogeny. Expression of the Lm-PHB2 protein was nearly equivalent in the heart, liver, kidneys, intestines, and muscles of normal lampreys. However, the Lm-PHB2 protein was down-regulated in the myocardia of lampreys challenged for 5 days with adriamycin (Adr), followed by a significant up-regulation 10 days after treatment. In vitro, recombinant Lm-PHB2 (rLm-PHB2) protein could significantly enhance the $H_2O_2$-induced oxidative stress tolerance in Chang liver (CHL) cells. Further mechanism studies indicated that the nucleus-to-mitochondria translocation of Lm-PHB2 was closely involved in the oxidative stress protection. Our results suggests that the strategies to modulate Lm-PHB2 levels may constitute a novel therapeutic approach for myocardial injury and liver inflammatory diseases, conditions in which oxidative stress plays a critical role in tissue injury and inflammation.



## 1. Introduction

Prohibitin 2 (PHB2) is a member of a protein superfamily that contains structurally related PHB domains and includes PHB1, stomatin, HflKC, and flotillin [1]. PHB1 and PHB2 have molecular masses of 32 and 37 kDa, respectively. PHB2, first identified as a repressor of estrogenic activity (REA) in mammalian cells [2], is a highly conserved, ubiquitously expressed protein, and homologues are found in bacteria, yeast, plants, drosophila, and mammals [3]. PHB2 interacts with the oestrogen receptor as well as with HDAC1 [4], appears to be essential for cell survival in eucaryotes [5] and has multiple functions, including the regulation of transcription and development and cell viability [6]. Previous reporter assays have demonstrated that PHB2 plays a critical role in the regulation of cell cycle progression through its interaction with E2F, pRb, and p53 [7–9], and PHB2 is aberrantly expressed in cancers such as neuroblastoma [10], breast cancer [11], liver cancer [12], ovarian cancer [13], and thyroid cancer [14]. In addition, knockdown of the Prohibitin 2 gene leads to the loss of PHB2 membrane scaffolds and impaired mitochondrial architecture, followed by Tau hyperphosphorylation and neurodegeneration [15].

Prohibitins are novel anti-proliferative proteins that play roles in cellular senescence, apoptosis, and the maintenance of mitochondrial function in mammals and yeast [16,17]. The name Prohibitin refers to the negative effects of these proteins on cell proliferation [1]. Prohibitins form large ring complexes in the inner membrane that are composed of prohibitin family members PHB1 and PHB2 and are thought to function as membrane scaffolds [18,19], and increasing evidence indicates that PHB2 may be a potential target for new therapeutics [20,21]. Considering the significant consequences of mitochondrial dysfunction in neurodegeneration and aging, an understanding of the molecular mechanisms of mitochondrial–nuclear communication that occurs through protein shuttling is crucial [22]. Mammalian PHB1 proteins mainly localise to the mitochondria and nucleus [5,10,23]. PHB1 translocates from the mitochondria to the nucleus during $H_2O_2$-induced oxidative stress in ARPE-19 cells [24], and PHB2

*Abbreviations:* PHB2, prohibitin 2; CHL cell, Chang liver cell; Adr, adriamycin; SOD, superoxide dismutase; Prx2, peroxiredoxin2; LDH, lactate dehydrogenase; XOR, xanthine oxidoreductase.

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translocation from the mitochondria to nucleus has also been observed in HeLa [25,26] and MCF-7 cells [4,8]. Translocation in the opposite direction, from the nucleus to mitochondria, was observed for PHB1 in INS-1E and RINm5F cells during ethanol-induced oxidative stress, which also increased PHB expression [27]. Although human PHB2 has pleiotropic functions in the mitochondria, including the inhibition of apoptosis and regulation of mitochondrial morphology, the molecular functions of PHBs, particularly PHB2, in the mitochondria during oxidative stress remain unclear.

The unique evolutionary position of lampreys, one of the most ancient vertebrates still alive today, makes them an ideal animal model for studying vertebrate evolution, embryo development, and the origin of adaptive immunity [28,29]. In contrast to the extensive studies on PHB2 in jawed vertebrates, little is known about PHB2 and its biological activities and physiological roles in jawless lampreys. In the present study, we report for the first time the molecular cloning and characterisation of a PHB2 homologue from Chinese northeast lamprey (*Lampetra morii*). We examined Lm-PHB2 expression in vital tissues and determined that Lm-PHB2 expression was altered in different tissues after adriamycin (Adr) treatment, which is used to establish oxidative stress environments for myocardia in myocardial disease research [30], with a significant decrease only in the liver. As human Chang liver cells are important normal cell materials used widely in liver stress and damage research [31], these cells were an appropriate choice for our ensuing studies. We found that recombinant Lm-PHB2 protected these cells from oxidative stress and that Lm-PHB2 was translocated from the nucleus to the mitochondria, thus reinforcing human PHB2 in the mitochondria in response to oxidative stress. These results emphasise the importance of elucidating the anti-oxidative and stress-related functions and evolution of the PHB2 protein.

Prohibitin exerts beneficial effects on neurons by reducing mitochondrial free radical production, and this protective effect of prohibitin has potential translational relevance for diseases of the nervous system that are associated with mitochondrial dysfunction and oxidative stress [32]. Gene silencing of PHB in epithelial cells induces mitochondrial autophagy via increased intracellular ROS [33]. Although moderate ROS can be useful for implementation of the immune system defence function [34], immune cells are more vulnerable to damage than other cells because of the fragility of the cell membrane when oxidative stress occurs [35]. Because oxidative stress induced by hydrogen peroxide has been implicated in many ocular diseases, we evaluated whether changes in Lm-PHB2 expression during $H_2O_2$ stimulation enhances cellular oxidative stress tolerance.

## 2. Materials and methods

### 2.1. Animals

Adult Chinese northeast lampreys (*L. morii*) (male and female, average weight of approximately 140 g) were obtained in December 2011 from the Yalujiang Valley of the Songhua River, Heilongjiang Province, China. For the oxidative stress study, a Chinese northeast lamprey was injected in the thoracic cavity with 1 ml of 200 μg/ml Adr (Sigma, USA) each day; the Adr was diluted in 0.9% physiological saline. Control animals were injected with 1 ml of 0.9% physiological saline during the same time frame. There were three animals in each group.

### 2.2. Cloning of lamprey PHB2

Total RNA was isolated from lamprey tissue using RNAiso Plus (TaKaRa, China). Reverse transcription was performed with 1 mg of total RNA, 50 pmol of oligo$(dT)_{18}$ primer, and 100 U of Moloney Murine Leukaemia Virus reverse transcriptase (M-MLV; TaKaRa, China) according to the manufacturer's instructions. Primers corresponding to the sequence of the 5′-UTR region of the sea lamprey PHB2 gene (Ensembl:ENSPMAG00000005886) were used to clone a cDNA encoding the PHB2 protein of the Chinese northeast lamprey by RT-PCR. The 3′ end of the full-length cDNA was identified by 3′RACE (rapid amplification of cDNA ends) using the 3′-Full Race Core Set with PrimeScript RTase (TaKaRa, China), and the full-length cDNA was named Lm-PHB2. The PCR product was sequenced, and the identity of the PHB2 sequence was verified using the Basic Local Alignment Search Tool (BLAST) of NCBI. The PCR product was purified, cloned into the pMD18-T vector using a DNA Ligation kit (TaKaRa, China), and transformed into *E. coli* DH5α cells. DNA sequencing was conducted with M13 Forward/Reverse primers using an ABI PRISM™ 3730XL DNA Analyzer (Applied Biosystems, USA) Table 1.

### 2.3. Amino acid sequence analysis

The predicted amino acid sequence of Lm-PHB2 was analysed using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The conserved domains were analysed at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. The signal peptide was predicted with http://www.cbs.dtu.dk/services/SignalP/.

### 2.4. Sequence alignment and construction of a phylogenetic tree

A total of 30 PHB1 and PHB2 sequences from lamprey and other species were obtained from NCBI (http://www.ncbi.nlm.nih.gov/Blast.cgi), and multiple sequence alignments were performed using BioEdit. The programs ClustalX and MEGA5 were used to construct the phylogenetic tree. The accession numbers of the PHB1 and PHB2 amino acid sequences extracted from the NCBI database are as follows: PHB1 — *Homo sapiens* (NP_001268644.1), *Pan troglodytes* (NP_001233332.1), *Canis lupus familiaris* (NP_001103649.1), *Bos taurus* (NP_001029744.1), *Mus musculus*

**Table 1**
Primers.

| Name | Sequence |
|---|---|
| **Cloning of the full-length cDNA** | |
| 3′RACE of Lm-PHB2 | |
| Outer primer | 5′-TACCGTCGTTCCACTAGTGATTT-3′ |
| Inner primer | 5′-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3′ |
| GSP1 | 5′-GCAGCTGGGCCTGGACTACGAG-3′ |
| GSP2 | 5′-GTGCTGCCCTCCATCGTCAA-3′ |
| CDS of Lm-PHB2 | |
| PHB2(upstream) | 5′-CGCTGGTGGTTTAGGTTTTC-3′ |
| PHB2(downstream) | 5′-TAGGCTACACCTCGGGACTT-3′ |
| **Cloning of recombinant protein** | |
| PHB2(upstream) | 5′-GGAATTCCATGGCTCAGCTCAAGGA-3′ |
| PHB2(downstream) | 5′-CCCAAGCTTGGGCTTCTTTTTCACCGAC-3 |
| **Analysis by semi-quantitative RT-PCR** | |
| PHB2(upstream) | 5′-CGCTGGTGGTTTAGGTTTTC-3′ |
| PHB2(downstream) | 5′-TAGGCTACACCTCGGGACTT-3′ |
| GAPDH(upstream) | 5′-CGTGCTGCCGTGCAAAAGGAA-3′ |
| GAPDH(downstream) | 5′-TTGGGAGTCGGCACGCGGAAC-3′ |
| **Analysis by real-time quantitative PCR** | |
| PHB2(upstream) | 5′-CCTCGCCTACGCTGCTAA-3′ |
| PHB2(downstream) | 5′-GGATAATGGAACCAGGGAAT-3′ |
| CAT(upstream) | 5′-CCGAAACTACCAGCGTGAC-3′ |
| CAT(downstream) | 5′-GGCACTGACGGCGAAT-3′ |
| GAPDH(upstream) | 5′- AACCAACTGCCTGGCTCCT -3′ |
| GAPDH(downstream) | 5′-GTCTTCTGCGTTGCCGTGT -3′ |
| **Construction of eucaryotic expression vector** | |
| PHB2(upstream) | 5′-GGAATTCCATGGCTCAGCAGCTC-3′ |
| PHB2(downstream) | 5′-CGGGATCCCGCTTCTTTTTCAC-3′ |