Discovery of conventional prolactin from the holocephalan elephant fish, *Callorhinchus milii*.

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Abstract

The conventional prolactin (PRL), also known as PRL1, is an adeno-hypophysial hormone that critically regulates various physiological events in reproduction, metabolism, growth, osmoregulation, among others. PRL1 shares its evolutionary origin with PRL2, growth hormone (GH), somatolactin and placental lactogen, which together form the GH/PRL hormone family. Previously, several bioassays implied the existence of PRL1 in elasmobranch pituitaries. However, to date, all attempts to isolate PRL1 from chondrichthyans have been unsuccessful. Here, we cloned PRL1 from the pituitary of the holocephalan elephant fish, *Callorhinchus milii*, as the first report of chondrichthyan PRL1. The putative mature protein of elephant fish PRL1 (cmPRL1) consists of 198 amino acids, containing two conserved disulfide bonds. The orthologous relationship of cmPRL1 to known vertebrate PRL1s was confirmed by the analyses of molecular phylogeny and gene synteny. The *cmPRL1* gene was similar to teleost *PRL1* genes in gene synteny, but was distinct from amniote *PRL1* genes, which most likely arose in an early amphibian by duplication of the ancestral *PRL1* gene. The mRNA of cmPRL1 was predominantly expressed in the pituitary, but was considerably less abundant than has been previously reported for bony fish and tetrapod PRL1s; the copy number of cmPRL1 mRNA in the pituitary was less than 1% and 0.1% of that of GH and pro-opiomelanocortin mRNAs, respectively. The cells expressing cmPRL1 mRNA were sparsely distributed in the *rostral pars distalis*. Our findings provide a new insight into the studies on molecular and functional evolution of PRL1 in vertebrates.

Keywords: prolactin; GH/PRL family; cartilaginous fish; molecular evolution

1. Introduction

Prolactin (PRL) is an adenohypophysial hormone produced in the anterior pituitary gland and secreted into circulation in response to various physiological stimuli. While PRL was originally named for its ability to stimulate lactation in rabbit (Stricker and Grueter, 1928; Riddle et al., 1933), it is a versatile hormone, exerting over 300 biological functions in mammalian and non-mammalian vertebrates, as reviewed by Bole-Feysot et al. (1998). In teleosts, PRL plays a critical role in maintaining plasma homeostasis in freshwater by altering salt- and water permeability across epithelial cell membranes in the gill, gut and kidney (see Hirano, 1986; Manzon 2002; Breves et al., 2014; Takei et al., 2014).

PRL shares its structural features with growth hormone (GH), bony fish somatolactin (SL) and mammalian placental lactogen (PL); these hormones form a monophyletic hormone family called GH/PRL family (see Rand-Weaver and Kawauchi, 1993). The origin and evolution of this hormone family has attracted the attention of researchers because of their diverse and important activities in organisms. Among the members of the GH/PRL family, GH is the only molecule found in all vertebrate classes including sea lamprey, a jawless fish (Kawauchi et al., 2002), suggesting that the rest of this hormone group arose from the ancestral *GH* gene via multiple gene duplications and subsequent evolutionary diversification. Meanwhile, the timing of emergence of current GH/PRL family members from ancestral GH remains to be explored. In previous studies, extensive efforts have been made to isolate PRL from the lineages of bony fish (osteichthyans) and cartilaginous fish (chondrichthyans). However, this was only successful in the bony fish lineages; consequently, the existence of PRL in cartilaginous fish remains uncertain. The extract of cartilaginous fish pituitary showed positive activity in the red eft water-drive test, implying the presence of PRL (see Bern and Nicoll, 1968). In Atlantic stingray, *Dasyatis sabina*, lesion of the *rostral pars distalis* (RPD) caused a significant increase in plasma osmolality, as well as plasma urea

and sodium levels; these effects were reversed by the injection of ovine PRL (de Vlaming et al., 1975). Pituitary PRL activity in the stingray was also investigated using the *Gillichthys* xanthophore assay, where the putative activity of pituitary PRL was upregulated approximately 100-fold by 24 h following transfer of stingrays from seawater (SW) to brackish water (de Vlaming et al., 1975). However, neither immunoreactive signal nor cDNA fragment was obtained for chondrichthyan PRL, despite the use of various heterologous PRL antisera and primers designed for conserved sequences of already identified PRL mRNAs (see Kawauchi and Sower, 2006).

A breakthrough in chondrichthyan biology was achieved by Venkatesh and colleagues, who initiated whole genome sequencing for the holocephalan elephant fish (or elephant shark, *Callorhinchus milii*) (<http://esharkgenome.imcb.a-star.edu.sg/>) (Venkatesh et al., 2007). We have focused on this species as a model for molecular endocrinological and physiological studies of cartilaginous fish (Hyodo et al., 2007; Kakumura et al., 2009; Yamaguchi et al., 2012; Takagi et al., 2014). Recently, a PRL-like gene was found from the elephant fish genome and designated PRL2 (Huang et al., 2009). Orthologs of the PRL2 gene exists throughout non-mammalian vertebrates, and phylogenetically PRL2 is distinct from the conventional PRL, which was subsequently renamed PRL1. This finding implies that the duplication of ancestral GH/PRL family gene occurred before the chondichthyan-osteichthyan divergence, and offers the possibility that a gene encoding PRL1 also exists in cartilaginous fishes.

In January 2014, the genome project for the elephant fish led to more comprehensive genome sequences (Venkatesh et al., 2014). In the whole genome sequences, we finally identified the conventional PRL, PRL1, of the elephant fish and cloned its cDNA. The elephant fish PRL1 mRNA was predominantly detected in the RPD of the pituitary as reported in other vertebrates, while the number of cells expressing PRL1 mRNA was extremely small compared with that in pituitaries of bony

fishes and tetrapods. Our analyses on gene synteny and molecular phylogeny bring a new insight into the molecular evolution of vertebrate GH/PRL family.

2. Materials and methods

2-1. Animals

Elephant fish, *Callorhinchus milii*, were collected in Western Port Bay, Victoria, and in Pitt Water, Tasmania, Australia. They were kept in 2,000 to 10,000 L round tanks filled with running SW under a natural photoperiod. In tissue sampling, the fish were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, St Louis, MO, USA). Tissues for RNA extraction were immediately frozen in liquid nitrogen. For *in situ* hybridization, the whole brain was dissected and fixed in Bouin's solution without acetic acid at 4°C for 2 days, and processed as described below.

All experiments were performed according to the Guideline for Care and Use of Animals approved by the committees of University of Tokyo, Deakin University, and University of Tasmania.

2-2. cDNA synthesis and Molecular cloning

The amino acid sequences of bony fish PRL and GH were used as BLAST queries to find candidate gene fragments in the Elephant Shark Genome Database (<http://esharkgenome.imcb.a-star.edu.sg/>), and the sets of specific primers were designed to amplify the transcripts of target genes. Total RNA was extracted from frozen tissue with Isogen (Nippon Gene, Tokyo, Japan) and treated by Turbo DNA-free kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Two µg of RNA was used to synthesize the first-strand cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies). The cDNAs encoding whole coding region of putative elephant fish PRL1 (cmPRL1) and partial coding region of

pro-opiomelanocortin (cmPOMC) were amplified with KAPATaq EXtra (Kapa Biosystems, Wilmington, MA, USA). For GH (cmGH), 5' - and 3' -RACE were performed to cover whole coding region using SMART cDNA library construction kit (Clontech, Mountain View, CA, USA). The amplified cDNA fragments were subcloned into pGEM T-easy (Promega, Madison, WI, USA) and their nucleotide sequences were determined by an automated DNA sequencer (3130xl Genetic Analyzer; Life Technologies). All primers used are listed in Table 1.

2-3. Molecular phylogenetic analysis

Protein sequences of PRL1, PRL2, GH and SL were retrieved from NCBI Genpept and Ensembl databases using aLeaves (Kuraku et al., 2013), and from coelacanth genome database (assembly ID; LatCha_J1.0, <http://coelacanth.nig.ac.jp/>) by BLAST search. The retrieved sequences were aligned with the alignment editor XCED in which the multiple sequence alignment algorithm is implemented (Katoh et al., 2013). Using amino acid sites unambiguously aligned, molecular phylogenetic trees were inferred preliminarily with the neighbor-joining (NJ) method using XCED and finally with the maximum-likelihood (ML) method using PhyML version 3.0 (Guindon et al., 2010). Among-site rate heterogeneity was taken into account by assuming the gamma distribution with the JTT model (JTT+ Γ_4 +I). In both NJ and ML methods, bootstrap resampling was performed with 100 replicates.

The GenBank accession IDs of sequences used in the analysis are listed in Supplementary Table 1. The amino acid sequences of putative PRL1s of Mexican salamander, *Ambystoma mexicanum*, were obtained by BLAST searches against their genome database at Sal-Site (assembly V3.0, <http://www.ambyostoma.org/>), using the sequences of *Xenopus laevis* proteins as queries.

2-4. Tissue distribution

Tissue distribution of cmPRL1 mRNA was examined by RT-PCR and quantitative real-time PCR (qPCR), using cDNAs prepared from each tissue as described above. RT-PCR was performed with KAPATaq EXtra (Kapa Biosystems) for 35 (cmPRL1) or 30 cycles (β -actin, as an internal control). Amplified PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide fluorescence.

The absolute quantification of target mRNAs was performed by qPCR with an ABI Prism 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA) and KAPA SYBR FAST ABI Prism qPCR Kit (Kapa Biosystems), as previously described (Takagi et al., 2014). The results were shown either as normalized by β -actin or as the copy number of target mRNAs per gram of RNA. All primers used are listed in Table 1.

2-5. *in situ* hybridization

The fixed brain tissue was washed twice in 70% ethanol and a small block of tissue containing the hypothalamus and pituitary was dissected out. The pituitary block was then embedded in Paraplast (Kendall, Mansfield, MA, USA) and sagittally sectioned. The sections of 6 μ m thickness were mounted onto MAS-GP-coated glass slides (Matsunami, Osaka, Japan)

The partial fragments of cmGH, cmPRL1 and cmPOMC transcripts were amplified using gene-specific primers listed in Table 1, and the PCR products were subcloned into pGEM-T easy (Promega). Digoxigenin (DIG)-labeled anti-sense cRNA probes were synthesized with DIG RNA Labeling Kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's protocols. The localization of target mRNAs in the brain was visualized by *in situ* hybridization with the cRNA probes mentioned above, as previously described (Takabe et al., 2012). Dual *in situ* hybridization was performed as described by Kanda et al. (2013), with minor

modifications, using the DIG-labeled probe for cmPRL1 and fluorescein-labeled probe for cmPOMC, which was synthesized using Fluorescein RNA Labeling Mix (Roche Applied Science). In brief, deparaffinized sections were digested by 5 µg/mL proteinase K (Wako Pure Chemical Industries, Osaka, Japan) and hybridized with 0.25 µg /mL probes at 58°C for 40h. Following immunohistochemical reactions with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) and horseradish peroxidase-conjugated anti-fluorescein antibodies (PerkinElmer, Waltham, MA, USA) at 4°C overnight, hybridization signals were visualized using Fast Red Tablets (Roche Applied Science) and TSA Plus Fluorescein System (PerkinElmer) according to the manufacturer's instruction. To ascertain the specificity of the signals obtained, sense cRNA probes were used as negative controls.

2-6. Synteny analysis

Synteny analyses were performed using UCSC Genome Browser (<http://genome.ucsc.edu/>), Ensembl Genome Browser (<http://www.ensembl.org/>) and NCBI database (<http://www.ncbi.nlm.nih.gov/>), as previously described (Yamaguchi et al., 2012). Firstly, the loci of candidate genes encoding GH/PRL family molecules were determined in elephant fish (*C. milii*), stickleback (*Gasterosteus aculeatus*), zebrafish (*Danio rerio*), coelacanth (*Latimeria chalumnae*), frog (*X. tropicalis*), chicken (*Gallus gallus*), and human (*Homo sapiens*), using the genome browsers mentioned above. Subsequently, 3-27 genes adjacent to the each target gene were identified using the NCBI RefSeq database and Ensembl Genome Browser. For genes not registered in NCBI reference sequence database, the deduced amino acid sequences of encoded proteins were subjected to NCBI TBLASTN searches for verification. Non-protein coding genes and genes coding unknown proteins were excluded from further analysis. Then, the orthologs of the identified genes were comprehensively searched in all examined species using the genome browsers and verified by NCBI TBLASTN

searches, when needed, as mentioned above. Finally, information on the loci of all identified genes were sorted and organized in figures. The conserved syntenic gene blocks were manually detected and compared among target genes.

2-7. Presentation of data and statistical analyses

Quantitative data are presented as means $\pm$ SEM. The results of qPCR were analyzed by Student's *t*-test or one-way ANOVA followed by Tukey's HSD test, as described in a figure legend. The data were log-transformed to satisfy normality and homogeneity of variance requirements, when necessary. *P* values less than 0.05 were considered statistically significant. All statistics were performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA, USA).

3. Result

3-1. Cloning

A BLAST search against the updated elephant fish genome database yielded fragments homologous to bony fish GH and PRL1, in addition to the previously identified PRL2. Subsequently, the cDNAs encoding putative GH (cmGH, GenBank accession ID LC055147) and PRL1 (cmPRL1, GenBank accession ID LC055146) were cloned from the elephant fish pituitary and sequenced. Alignment of cloned cDNAs with elephant fish genome revealed that the coding regions of both cmGH and cmPRL1 consist of five exons (Fig. 1). The putative mature protein of cmPRL1 is composed of 198 amino acid residues, containing six cysteine residues (at positions 19, 60, 161, 173, 190 and 198). Four out of six Cys residues (Cys⁶⁰, Cys¹⁷³, Cys¹⁹⁰ and Cys¹⁹⁸) were conserved among all GH/PRL family molecules, indicating that mature cmPRL1 forms two disulfide bridges (Fig. 1); on the other hand, the PRL1s of sturgeon, coelacanth, lungfish and tetrapods contain two more conserved Cys residues in amino-terminus

(N-terminus) forming an additional disulfide bond. A putative N-glycosylation site was detected at Asn⁶². The carboxyl-terminal (C-terminal) region of cmPRL1 was highly homologous to known PRL1 proteins and also to other GH/PRL family members (Fig. 1). The Pro⁹⁶ and Gly¹³¹ of mature cmPRL1 were residues found to be well conserved among all GH/PRL family members; these residues are considered to be important in the binding of molecules to their receptors (Goffin et al., 1996; Schenck et al., 2003).

The putative mature cmGH protein is composed of 184 amino acid residues, showing high similarity to blue shark GH (76% identity). Alignment of cmGH with known GHs revealed four conserved Cys residues forming two intramolecular disulfide bridges (Cys⁵², Cys¹⁵⁷, Cys¹⁷⁴ and Cys¹⁸²) in all GH proteins compared (Fig. 1).

Regarding the conserved Pro and Gly residues described above, the Pro residue was replaced by the Ser⁸⁸ in mature cmGH, whereas the Gly residue was found as Gly¹¹¹.

The Thr-Val residues were extended in the C-terminal end after Cys¹⁸², as another structural feature of GH molecules. The amino acid identity of cloned molecules to representative vertebrate PRL1s and GHs is shown in Table 2.

3-2. Molecular phylogenetic analysis

The phylogenetic relationship between the identified elephant fish genes and known GH/PRL family hormones was investigated by constructing a molecular phylogenetic tree. The tree was inferred with the maximum-likelihood method using 114 amino acid sites unambiguously aligned (Fig. 2). The resulting tree consisted of four separate monophyletic clades of PRL1, PRL2, GH and SL; cmPRL1 and cmGH were grouped into the PRL1 and GH clades, respectively. A notable finding was that at least two genes belonging to the PRL1 clade were detected in all examined amphibians. Phylogenetic relationships within the PRL1 clade were further investigated using 174 amino acid sites (Fig. 3). In *X. laevis*, three PRL proteins have been reported (GenBank accession IDs NP_001086486, AAH92151, and NP_001159915), and all of them were

grouped in the PRL1 clade, but not in the PRL2 clade (Figs. 2 and 3). For these PRLs of *X. laevis*, we follow the nomenclature suggested by Huang et al (2009): xIPRL1A for NP_001086486 and xIPRL1B for NP_001159915. Since a sister relationship was detected between NP_001086486 (xIPRL1A) and AAH92151, we propose to designate them as xIPRL1A1 and xIPRL1A2, respectively. The existence of multiple *PRL1* genes also became evident in *X. tropicalis* and urodele axolotl *A. mexicanum*. Two PRL proteins from *X. tropicalis*, one reported (GenBank accession ID AAI36078) and one predicted (GenBank accession ID XP_002938572), were orthologous to xIPRL1A and xIPRL1B, respectively (Fig. 3); the predicted amino acid sequence of XP_002938572 was identical to that of xIPRL1B. Similarly, in the genome of *A. mexicanum*, two putative genes encoding proteins orthologous to xIPRL1As and xIPRL1B were found on contig_62377 and contig_27042 (Sal-Site), respectively. These data suggest that the *PRL1* gene duplicated in an ancestral amphibian, generating *PRL1A* and *PRL1B* genes shared by both anurans and urodeles. In the phylogenetic tree, amphibian PRL1As form a sister group of amniote PRLs, while PRL1Bs branched between the PRL1s of actinopterygian and sarcopterygian fish (Fig. 3).

3-3. Synteny analysis

The orthologous relationships between elephant fish *GH* and *PRL* genes and known GH/PRL family genes were examined by comparing location of genes adjacent to target genes, among the representative species of different vertebrate classes. Consistent with the molecular phylogenetic tree (Figs. 2 and 3), four GH/PRL family members (PRL1, PRL2, GH and SL) were distinguishable by their unique patterns of conserved synteny (Fig. 4; Supplementary Figs. 1-3). For *GH* gene, the syntenic gene block was well conserved throughout vertebrates and was identified on chromosome 17 in human (Supplementary Fig. 1).

In the case of *PRL1* gene, the organization of its neighboring genes were

completely different between fishes and tetrapods; *PRL1* genes of bony fishes (stickleback, zebrafish, and coelacanth) were adjacent to the orthologs of human genes on chromosome 19, while human *PRL1* gene locates on chromosome 6 showing a conserved syntenic pattern to tetrapod *PRL1* genes (Fig. 4). In *X. tropicalis*, two distinct *PRL1* genes identified in the molecular phylogenetic analysis, *PRL1A* and *PRL1B*, were found on separate genomic contigs. The gene blocks around *PRL1A* and *PRL1B* genes of *X. tropicalis* resembled those around amniote *PRL1* genes and bony fish *PRL1* genes, respectively. The elephant fish *cmPRL1* gene was identified on scaffold 350, where only three genes, *TRIM28*, *EMC10* and *ELF5*, were identified besides *cmPRL1* gene, due to a captured gap spanning nearly 60% of the scaffold length. Similar colocalization of *ELF5* gene with *PRL1* gene on the same chromosome was also detected in stickleback and zebrafish; this implies that the gene order around *cmPRL1* gene resembles that around teleost *PRL1* and *X. tropicalis* *PRL1B* genes, rather than that around *X. tropicalis* *PRL1A* and amniote *PRL1* genes.

Although *PRL2* gene was not detected in human and amphibians, its synteny pattern was well conserved from bony fish to tetrapods, being found on human chromosome 13 (Supplementary Fig. 2). In the elephant fish, unlike *PRL2* genes of other species, *cmPRL2* gene was located together with the orthologs of human genes on chromosome 2 and 21. In the case of bony fish *SL* gene, the syntenic gene block was identified on human chromosome 11 (Supplementary Fig. 3). Despite the conserved gene synteny throughout the vertebrate species, *SL* gene was found only in bony fishes, but not in the elephant fish and tetrapods.

3-4. Tissue distribution of mRNAs

The tissue distribution of *cmPRL1* mRNA was investigated by RT-PCR (Fig. 5). Among the examined tissues, *cmPRL1* mRNA was predominantly expressed in the pituitary. Brain levels were considerably lower compared with those in the pituitary.

Subsequently, mRNA expression of cmPRL1, cmGH and cmPOMC in the pituitary and other brain tissues were further verified by qPCR (Fig. 6). Consistent with RT-PCR results, mRNA expression of PRL1, as well as that of cmGH and cmPOMC, was highest in the pituitary (Fig. 6A-C). The copy number of cmPRL1 mRNA in the pituitary was less than 1% of that of cmGH mRNA and 0.1% of that of cmPOMC mRNA (Fig. 6D).

3-5. Localization of mRNAs of cmPRL1, cmGH and cmPOMC in the pituitary

The spatial distribution of cmPRL1 mRNA in the pituitary was investigated by *in situ* hybridization (Fig. 7). Hybridization-positive cells for cmPRL1 mRNA were sparsely distributed in the RPD. A few positive cells were found in the *posterior pars distalis* (PPD) as well (Fig. 7A, B and C). Most RPD cells, as well as cells in the *pars intermedia* (PI), were intensely stained with the antisense cRNA probe for cmPOMC mRNA (Fig. 7A, B and D). Dual staining with the probes for cmPRL1 and cmPOMC showed that PRL1 mRNA and POMC mRNA are expressed in separate RPD cells (Fig. 8). The signals for cmPOMC mRNA were also detected in PPD and basal hypothalamic neurons at lower densities (Fig. 7A and C, Supplementary Fig. 4). On the other hand, cmGH mRNA was abundant in the PPD (Fig. 7A and C), while it was also expressed in some RPD cells (Fig. 7B). For all target mRNAs, hybridization with sense cRNA probes did not elicit any positive signal (data not shown), confirming the specificity of the signals obtained with antisense probes.

4. Discussion

4-1. Discovery of PRL1 from the elephant fish

In the present study, we identified and successfully cloned the cDNA of PRL1, the conventional PRL, from the elephant fish, as the first report of this hormone in

chondrichthyans. The orthologous relationship between *cmPRL1* gene and known *PRL1* genes was confirmed by molecular phylogenetic analysis and gene synteny analysis. Together with previously reported *cmPRL2* and newly cloned *cmGH*, our findings revealed that the molecular diversification in GH/PRL family occurred at least, in the chondrichthyans, the earliest-diverged group of living gnathostomes.

The existence of PRL1 in chondrychtyans was implied by the red eft water-drive test using the pituitary extracts from cartilaginous fish (see Bern and Nicoll, 1968). In Atlantic stingray, plasma osmolality, sodium and urea concentrations were significantly upregulated following the lesion of RPD, while the effects of RPD-lesion were reversed by the injection of oPRL (de Vlaming et al., 1975). However, subsequent attempts over many years to isolate chondrichthyan PRL1 protein or PRL1 cDNA were unsuccessful. Consistent with the previous reports, *cmPRL1* mRNA was predominantly expressed in the RPD of the elephant fish pituitary. Prior failures in searching for chondrychthyan PRL1 were most probably due to the considerably small population of PRL1-producing cells scattered in the RPD. Although we did not quantify the number of elephant fish RPD cells expressing *cmPRL1* mRNA, they appeared to be less than 10% of cells expressing POMC mRNA. This observation was further supported by the qPCR analysis on whole pituitary; the quantity of *cmPRL1* mRNA was less than 1% of *cmGH* mRNA and 0.1% of *cmPOMC* mRNA. Meanwhile, PRL1-producing cells account for 10-25% of human pituitary cells; this is the second largest cell population in the human pituitary after that of somatotrophs (40-50%) and comparable with that of corticotrophs (15-20%) (Nussey and Whitehead, 2001). In teleost fish pituitaries, PRL1-producing cells occupy a large homogenous mass in the RPD, making a clear contrast with the elephant fish pituitary (see Ball and Baker, 1969; Holmes and Ball, 1974).

4-2. Evolution of GH/PRL family

Comparison of the deduced amino acid sequences of elephant fish proteins with those of the known PRLs, GHs and SLs revealed some structural features. First, the C-terminal region, in particular the residues between Cys¹⁷³ and Cys¹⁹⁰ (positions in mature cmPRL1) is well conserved among all examined GH/PRL family molecules. In cmPRL1, Pro⁹⁶ and Gly¹³¹, the residues which significantly contribute to the receptor binding ability of the molecules (Goffin et al., 1996; Schenck et al., 2003), were conserved between known PRL1s. Although the C-termini of cmPRL1 and known PRL1 proteins terminate with the conserved Cys residue, the cmGH has the extension of two amino acid residues after Cys¹⁸² in its C-terminal end; such an extension of C-terminal residues is a feature of known GHs and SLs.

The most prominent structural characteristic of GH/PRL family is the conserved intramolecular disulfide bonds. All reported SLs, PRL2s (except for cmPRL2) and tetrapod PRL1s contain three disulfide bonds, while GHs and teleost PRL1s have only two disulfide bonds, lacking one in the N-terminus and a subsequent small loop structure (see Manzon, 2002; Rand-Weaver and Kawauchi, 1993; Sinha, 1995). These disulfide bonds are important clues to understand the evolutionary history of the GH/PRL family. Previous studies of primitive actinopterygians have provided valuable information. The sturgeon PRL1, as well as the sarcopterygian (coelacanth, lungfish and tetrapods) PRL1s, contains three disulfide bonds (Noso et al., 1993). Meanwhile, the PRL1 of holostean bowfin (*Amia calva*) does not have the disulfide bond in the N-terminus; the analysis of N-terminus of bowfin PRL1 and GH suggested that these proteins are more closely related to teleost PRLs and GHs than to those of tetrapods (Dores et al., 1993). Although basal actinopterygian relationships are still somewhat controversial (Inoue et al., 2003), molecular phylogenetic analyses using multiple nuclear gene sequences showed that holosteans (bowfin and gar) and teleosts share a common ancestor, forming a sister group of the order Acipenseriformes (sturgeons and paddlefishes) (Kikugawa et al., 2004; Near et al., 2012). This is

consistent with the currently accepted taxonomic classification, in which both holostean and teleost fish are grouped into the subclass Neopterygii (see Nelson, 2006). Based on these findings, Kawauchi and colleagues drew a schematic diagram of the molecular evolution of the GH/PRL family, postulating that an ancestor of the ray-finned fishes possessed PRL with three disulfide bonds, and that the N-terminal bond was lost throughout the evolution of teleosts (Dores et al., 1993; Kawauchi and Sower, 2006; Noso et al., 1993). In this context, it is of great interest that cmPRL1, as well as previously reported cmPRL2, lacks the N-terminal disulfide bond; so far, the N-terminal disulfide bond has not been found in any of GH/PRL family proteins in cyclostomes and cartilaginous fish. Further studies are required to clarify when the N-terminal intramolecular disulfide bond was acquired and lost along the divergence of the GH/PRL family.

The present synteny analyses offered a new insight into the evolution of *PRL1* gene. In the case of *GH* gene, the gene synteny around it was well conserved throughout vertebrates, from elephant fish to human. On the other hand, the gene order around *PRL1* gene was completely different between fishes and tetrapods. For bony fish PRL1, the orthologous gene block was found on human chromosome 19, while that for tetrapod PRL1 was identified on human chromosome 6. This discrepancy was solved by the existence of two *PRL1* genes in amphibians, designated as *PRL1A* and *PRL1B*, respectively. The *X. tropicalis* *PRL1B* gene showed a conserved synteny with fish *PRL1* genes, while conservation of gene order was identified between the regions containing *X. tropicalis* *PRL1A* gene and amniote *PRL1* genes. The observed gene synteny patterns, together with the molecular phylogenetic trees, suggest that the ancestral *PRL1* gene duplicated in an early amphibian to generate these two *PRL1* genes. After the duplication of *PRL1* gene in amphibians, the original *PRL1B* gene was lost and the newly arisen *PRL1A* gene was inherited by amniotes. Similar to the distinct gene syntenic patterns of fish and tetrapod *PRL1* genes, the gene blocks around *PRL2* gene

were not conserved between elephant fish and other species. A putative gene duplication of ancestral *PRL2* gene, which supposedly took place before the divergence of teleost lineages, may account for this inconsistency in gene synteny. Further studies in primitive vertebrates are required to test this hypothesis.

Among GH/PRL family molecules, SL has been reported to be unique to bony fish lineages (Amemiya et al., 1999; Ono et al., 1990; Rand-Weaver et al., 1991). Consistent with this observation, we could not find *SL* gene in the genome of either elephant fish or tetrapods, while the gene block around *SL* gene is extremely well conserved throughout vertebrates. In our molecular phylogenetic tree, PRL1 and SL were branched as sister groups of PRL2 and GH, respectively, suggesting that the current GH/PRL family was established via at least two gene (or genome) duplication events; in the first event, an ancestral gene, possibly *GH* gene, was duplicated into two lineages of PRL1/PRL2 and GH/SL, and subsequent gene duplications in each lineage formed the four distinct clades. Since three of the four members of the GH/PRL family were found in the elephant fish (cmPRL1, cmPRL2 and cmGH), at least the first duplication event and the subsequent diversification between PRL1 and PRL2 clades were completed before the chondrichthyan-osteichthyan split. So far, we do not have definitive evidence to conclude the exact timing of *SL* gene occurrence; it may have diverged at a very early stage of vertebrate evolution together with other GH/PRL family members, and secondarily lost in both chondrichthyans and tetrapods. Alternatively, the *SL* gene may have been acquired in the evolution of bony fishes, and then lost in the tetrapods.

4-3. Possible function of PRL1 in cartilaginous fish

Currently, the function of PRL1 in cartilaginous fish remains unclear. As seen with PRL1 in other vertebrates, the transcript of cmPRL1 was predominantly expressed in the RPD. The involvement of PRL1 in body fluid homeostasis has been well

established throughout vertebrates (Bole-Feysot et al., 1998). In teleost lineages, PRL1 is known as a key hormone in freshwater adaptation (see Manzon 2002). Hypophysectomized euryhaline killifish, *Fundulus heteroclitus*, fail to survive in fresh water; their ability to survive is restored by administration of ovine PRL (Pickford and Phillips, 1959). Similarly, the *in vivo* injection of oPRL to RPD-lesioned Atlantic stingray reversed the effects of surgery, in terms of plasma parameters (de Vlaming et al., 1975). We previously demonstrated, however, that the elephant fish can only survive in a relatively narrow window of salinities (Hyodo et al., 2007), suggesting that the major roles of cmPRL1 may not involve osmoregulation. This idea is further supported by our finding that PRL1 is produced in extremely small numbers of RPD cells in the stenohaline elephant fish pituitary. In euryhaline Mozambique tilapia, *Oreochromis mossambicus*, several PRL-related parameters are greater in fish acclimated to fresh water than that reared in seawater, such as the size of RPD and PRL1-producing cells (Dharmamba and Nishioka, 1968), pituitary content of PRL1 mRNA (Magdeldin et al., 2007) and protein (Nicoll et al., 1981), and circulating PRL1 level (Nicoll et al., 1981). Thus, it is of great interest to examine the dynamics of chondrichthyan PRL1 in the context of their distinct salinity tolerances; while most species of the class Chondrichthyes inhabit marine environments, stingrays of the family Potamotrygonidae spend all their lives in freshwater, and some species travel from the ocean to rivers, such as the bull shark and Atlantic stingray (see Ballantyne and Fraser, 2013; Martin, 2005). In Atlantic stingray, the putative PRL activity measured by *Gillichthys* xanthophore assay increased 100-fold following the transfer of stingrays from seawater to brackish water (de Vlaming et al., 1975). Future investigations on the levels of PRL1 gene expression and protein in the pituitary and circulation of cartilaginous fishes under a variety of salinity and other physiological conditions will help elucidate the functional evolution of PRL.

In addition to the aspect as an osmoregulatory factor, a putative contribution of

PRL1 in chondrichthyan reproduction is also of interest. PRL1 is indispensable in vertebrate reproduction by triggering numerous events such as mammalian lactation, avian crop sac growth (see Horseman and Buntin, 1995), hepatic vitellogenin synthesis in amphibians (Carnevali et al., 1993), steroidogenesis and gonadogenesis in teleosts (see Whittington and Wilson 2013), and parental behaviors in all these species (see Bole-Feysot et al., 1998; Horseman and Buntin, 1995; Polzonetti-Magni et al., 1995; Whittington and Wilson 2013). Cartilaginous fish are well known for their diverged reproductive strategies ranging from oviparity to various modes of viviparity (see Compagno, 1990; Wourms, 1977). The rays of the order Myliobatiformes, for example, adopt a form of reproduction called histotrophy, where "uterine milk" is secreted from the uterus for embryonic growth (see Compagno, 1990; Wourms, 1977). The endocrine regulation of their reproductive status, however, is largely unknown (see Awruch, 2003).

For understanding the function of PRL1 in cartilaginous fish, further attempts to determine and characterize its target receptor (PRLR) are indispensable. The PRLR exhibits a single chain structure with one transmembrane domain, and dimerizes upon binding to PRL1. While our preliminary search in the elephant fish genome sequences failed to find a gene potentially orthologous to known *PRLR* genes, the gene encoding putative GH receptor (GHR) was identified on scaffold 89 (GenBank accession ID XM_007901587). It is noteworthy that GHR belongs to the same receptor superfamily as PRLR and these receptors show considerable similarity both in structure and in signal transduction (see Freeman et al., 2000; Kopchick and Andry, 2000). The similarities between GH and PRL1 proteins and between their receptors allow GH to bind and signal via PRLR, and vice versa. Human GH is known to interact with both GHR and PRLR (Cunningham et al., 1990; Somers et al., 1994), and in Mozambique tilapia, one of two isoforms of PRL1 (PRL₁₇₇) was suggested to elicit somatotrophic effect via GHR (Shepherd et al., 1997). While our observation does not necessarily preclude PRLR in the elephant fish, the binding of cmPRL1 to GHR is worth exploring. Further

characterization of the GH/PRL molecules and their receptors in other chondrichthyans and cyclostomes will shed light into the missing links underling the evolutionary history of this classical hormone family.

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Figure legends

Figure 1. Multiple alignment of amino acid sequences of PRL/GH/SL. The alignment was constructed with the MAFFT algorithm (see Methods) and manually modified for improved demonstration of conserved residues as well as structural features unique to each hormone. Arrows; the positions of the introns of cmPRL1 (black) or cmGH (white), black circles; conserved cysteine residues, triangles; putative *N*-glycosylation site of cmPRL1 (black) or conserved proline and glycine residues important in receptor binding (white). Abbreviations: ag, *Acipenser gueldenstaedtii* (sturgeon); cm, *Callorhinchus milii* (elephant fish); dr, *Danio rerio* (medaka); gg, *Gallus gallus* (chicken); hs, *Homo sapiens* (human); lo, *Lepisosteus oculatus* (gar); paSL, *Protopterus annectens* (African lungfish) SL; paPRL1, *Protopterus aethiopicus* (Marbled lungfish) PRL1; xl, *Xenopus laevis* (frog). See Supplementary Table 1 for accession IDs of these sequences.

Figure 2. Molecular phylogeny of the entire PRL/GH family inferred using 114 amino acid sites (shape parameter of the gamma distribution $\alpha = 2.2$). The elephant fish

sequences identified in this study are shown in white letters with a black background. Individual gene names are given when they do not follow the basic subgrouping on right (PRL1, PRL2, GH, and SL). At nodes, bootstrap probabilities from the maximum-likelihood method and neighbor-joining method are shown in order. The arrow indicates the position of the branch leading to the sequence of *Petromyzon marinus* GH (GenBank accession ID, AB081461) in a preliminary phylogenetic analysis as reference.

Figure 3. Molecular phylogeny of the jawed vertebrate PRL1 inferred using 165 amino acid sites (shape parameter of the gamma distribution $\alpha=1.7$). At nodes, bootstrap probabilities the maximum-likelihood method and neighbor-joining method are shown in order. The gray diamond indicates gene duplication that gave rise to the two *PRL1* genes (*PRL1A* and *PRL1B*) in an early sarcopterygian. The white diamond indicates gene duplication unique to the *Xenopus* lineage. Note that the predicted amino acid sequence of *X. tropicalis* PRL1B was identical to that of *X. laevis*.

Figure 4. Gene synteny around *PRL1* genes of elephant fish (*C. milii*), stickleback (*G. aculeatus*), zebrafish (*D. rerio*), coelacanth (*L. chalumnae*), frog (*X. tropicalis*), chicken (*G. Gallus*), and human (*H. sapiens*). Gene loci on a chromosome (chr.) and scaffold (scf.) are shown by ellipses serially numbered in alphabetical order. The color of the ellipses represents the human chromosome on which corresponding human orthologs are found.

Figure 5. Gel images showing the tissue distribution of cmPRL1 and β -actin as an internal control. For non-gonadal tissues, left and right lanes represent the results of female and male, respectively. Abbreviations: Bra, brain; Pit, pituitary; Gil, gill; Int, intestine; Kid, kidney; Liv, Liver; RG, rectal gland; U, uterus; T, testis; M, molecular

weight marker.

Figure 6. (A-C) Quantification of mRNA of cmPRL1 (A), cmGH (B) and cmPOMC (C) in the elephant fish brain (Bra) and pituitary (Pit). The results are shown as normalized values by β -actin. ***Significantly different from brain at $P < 0.001$ (Student's t -test). (D) Absolute copy number of mRNA in the pituitary was compared among the above three targets. Note that Y axis is in logarithmic scale. Means not sharing the same letter are significantly different at $P < 0.01$ (Tukey's HSD test following one-way ANOVA).

Figure 7. Images of sagittally-sectioned elephant fish pituitary subjected to either hematoxylin-eosin (HE) staining or hybridization with cRNA probes for cmPRL1, cmPOMC or cmGH. (A) Whole pituitary images. Scale bar represents 1 mm. (B-C) Magnified images of RPD (B), PPD (C) and PI (D). Positive hybridization signals for probes of cmPRL1 in RPD (B) and PPD (C) and of cmPOMC in PPD (C) are indicated by arrowheads. Scale bars represent 0.5 mm.

Figure 8. Dual fluorescent *in situ* hybridization of the elephant fish pituitary for mRNAs of cmPRL1 (magenta) and cmPOMC (green). Areas enclosed by white line in top row are magnified in bottom row. Scale bars represent either 100 μ m (top row) or 50 μ m (bottom row), respectively. No overlap was observed between signals of cmPRL1 and cmPOMC mRNAs.

Table 1

Table 1. List of primers used in the present study. *For cmGH, forward and reverse primers were used for 3' - and 5' -RACE, respectively.

Cloning	Forward primer (5'-3')	Reverse primer (5'-3')
cmPRL1	gtcggacgagccgtggacact	cagaggggtgtcgagagatgc
cmGH*	acctcttgccaacgctgttcac	cttcttaggttccttcgaaccg
cmPOMC	agaccctgactgtcacctcaa	attcctgaagagggtgagcagt
RT-PCR		
cmPRL1	(same with the cloning primer)	tcagcaggacaccgcgtgagg
β-actin	aagacatcagggtgtgatggttg	ggagcaatgatcttgatcttcattg
qPCR		
cmPRL1	ctctgtccaacgacatcttcaa	acgggttcgtgccaatac
cmGH	ccggaagagaagcgtaactatc	agtacgcaagaagctctgtatc
cmPOMC	gcagaagaaggatgggaagt	gaacatgggaagcagatgtttag
in situ hybridization		
cmPRL1	(same with the cloning primer)	(same with the cloning primer)
cmGH	caggattgtcgctgtgttct	ggtaggtttcaactttgtgcatac
cmPOMC	(same with the cloning primer)	(same with the cloning primer)

Table 2. Amino acid identity of cmPRL1 and cmGH to representative vertebrate PRL1s and GHs.

PRL1

Species (GenBank accession ID)	% identity to cmPRL1
Sturgeon (AAB28396)	35%
Trout (M24738)	32%
Lungfish (AAB27569)	35%
Frog (CAA34199)	37%
Chicken (BAB18728)	37%
Human (NP_000939)	35%

GH

Species (GenBank accession ID)	% identity to cmGH
Sea lamprey (BAC15763)	27%
Shark (P34006)	76%
Sturgeon (AAX36064)	59%
Tilapia (M26916)	34%
Lungfish (AAC16496)	53%
Newt (CAB55428)	53%
Chicken (AEZ51530)	54%
Human (NP_00205)	42%

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

C

D

Figure 7

Figure 8