博士論文(要約)

# Studies on growth hormone as a regulator of lipid metabolism in torafugu *Takifugu rubripes*

(トラフグ脂質代謝制御因子としての成長ホルモンの機能解析)

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# Studies on growth hormone as a regulator of lipid metabolism in torafugu *Takifugu rubripes*

Yuki Hirano

Laboratory of Marine Biochemistry

Department of Aquatic Bioscience

Graduate School of Agricultural and Life Sciences

The University of Tokyo

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**List of abbreviations**

AAP : Abridged Anchor Primer

ANOVA : Analysis of variance

AUAP : Abridged Universal Amplification Primer

Akt : Protein kinase B

BLAST : Basic local alignment search tool

BN-PAGE : Blue-native polyacrylamide gel electrophoresis

BSA : Bovine serum albumin

CBB : Coomassie Brilliant Blue

CE : Cholesteryl ester

CM : Chylomicron

CPT1 : Carnitine palmitoyltransferase 1

CoA : Coenzyme A

DNA : Deoxyribonucleic acid

EDTA : Ethylenediaminetetraacetic acid

EF-1α : Elongation factor 1 alpha

ERK : Extracellular signal-regulated kinase

FAS : Fatty acid synthase

GH : Growth hormone

GHR : Growth hormone receptor

GO : Gene ontology

HDL : High-density lipoprotein

HPLC : High-performance liquid chromatography

HSL : Hormone sensitive lipase

IGF-I : Insulin-like growth factor-I

IRS : Insulin receptor substrate

JAK2 : Janus kinase 2

LDL : Low-density lipoprotein

LPL : Lipoprotien lipase

NEFA : Non-esterified fatty acid

ORF : Open reading frame

PAGE : Polyacrylamide gel electrophoresis

PBS : Phosphate-buffered saline

PCR : Polymerase chain reaction

PI3K : Phosphatidylinositol 3-kinase

PPAR : Peroxisome proliferator-activated receptor

RACE : Rapid amplification of cDNA ends

RNA : Ribonucleic acid

RT-PCR : Reverse transcribed PCR

SDS : Sodium dodecyl sulfate

STAT : Signal transducer and activator of transcription

TAG : Triachlglycerol

TBS : Tris-buffered saline

TBS-T : TBS-Tween 20

TCA : Tricarboxylic acid

TdT : Terminal deoxynucleotidyl transferase

Tris : Tris(hydroxymethyl)aminomethane

UTR : Untranslated region

VLDL : Very low-density lipoprotein

cDNA : Complementary deoxyribonucleic acid

dNTP : Deoxynucleotide triphosphate

mRNA : Messenger ribonucleic acid

pNPP : *p*-nitrophenyl phosphate

rtGH : Recombinant torafugu growth hormone

**Contents**

## General Introduction

Vertebrates have the ability to survive food deprivation by maintaining their energy metabolism. They can basically use carbohydrates, proteins, and lipids as energy sources. Because lipids contain more energy per gram than proteins and carbohydrates (van Dam and Seidell, 2007), they function principally as energy reserve. Vertebrates usually store lipids in adipose tissues that are distributed in several regions such as visceral, subcutaneous, and intramuscular region (Goodpaster and Wolf, 2004). In the case of fish, excess energy intake results in lipid deposition in specific tissues, such as adipose tissue, liver, and skeletal muscle (Sheridan, 1988). The distribution patterns of lipids among these organs are known to be species-specific. Torafugu (Japanese pufferfish) *Takifugu rubripes* accumulates lipids mainly in the liver but hardly in the skeletal muscle like Japanese flounder *Paralichtyhs olivaseus* and cod *Gadus morhua* (Ando et al., 1993; Santos et al., 1993). These fish, often referred to as "lean fish", usually have no mature adipose tissue. On the other hand, "fatty fish" species including red seabream *Pagrus major*, amberjack *Seriola dumerili*, and Japanese eel *Anguilla japonica* accumulates lipids in liver, skeletal muscle, and adipose tissues (Ando et al., 1993; Oku et al., 2009). Since the lipid contents of fish body affect its taste and the quality of final products, most previous studies on fish lipid metabolism have been conducted on the latter group. However, cross-species comparison between lean and fatty fish may also provide us with information about the similarities and differences of the process. It will enable us to understand more basic aspects of fish lipid metabolism. For these reasons, this study focused on the mechanisms of maintaining lipid metabolism in lean fish.

### *Stored lipid metabolism in fish*

The excess energy obtained by feeding is stored in the form of triacylglycerols (TAGs) in several tissues. TAG is a neural lipid consisting of a glycerol backbone bonded to three fatty acids

esterified, which are provided from blood circulation or synthesized *de novo* catalyzed by fatty acid synthase (FAS) (Tocher, 2003). When the energy demands are increased due to the fasting or exercise, stored TAGs are hydrolyzed by hormone sensitive lipase (HSL) to release free fatty acids (non-esterified fatty acids, NEFAs) as energy fuels (Nakamura et al., 2014). These resulting NEFAs are subsequently esterified to coenzyme A (CoA) to form fatty acyl-CoA, and transported into mitochondria to undergo β-oxidation. Carnitine palmitoyltransferase 1 (CPT1), localized on the mitochondrial outer membrane, is known as the rate-limiting enzyme of β-oxidation catalyzing fatty acyl-CoA transport into the mitochondrial matrix (Kerner and Hoppel, 2000). The fatty acyl chains derived from NEFAs are then oxidized to produce acetyl-CoA, the substrate of tricarboxylic acid (TCA) cycle for energy production. These enzymes, which play important roles in lipid metabolism, are identified in fish species with similar forms and functions as in mammals, suggesting the conservation of lipid metabolism among vertebrates.

Because of the hydrophobic nature of TAG, it forms lipid droplets for long-term storage. The distribution pattern of lipid droplets is tissue-specific in fish. In liver and adipose tissue, numerous lipid droplets are observed in cytoplasm of hepatocytes and adipocytes, respectively (Akiyoshi and Inoue, 2004; Oku and Umino, 2008), whereas in skeletal muscle, the adipocyte-like cells including abundant lipid droplets are localized along the myosepta (Tocher, 2003; Kaneko et al., 2013).

*Circulation of lipids and lipoproteins*

Body lipid mobilization is induced depending on the energy demand of peripheral tissues. Since TAGs, the typical lipids stored in tissues, are insoluble in water, they associate with apolipoproteins to form lipoprotein complexes (Murray et al., 2003). As in mammals, fish lipoproteins are classified into chylomicron (CM), very low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs) depending on the densities and particle sizes (Chapman et al., 1978; Babin and Vernier, 1989; Iijima et al., 1995). CM is a dietary-derived triacylglycerol (TAG)-rich

lipoprotein produced from intestine in the postprandial period. VLDL is a secondly TAG-rich lipoprotein released from liver, which has the function of TAG transport from liver to peripheral tissues. TAGs in these lipoproteins are incorporated into peripheral tissues by mainly two ways. One is enzyme-mediated incorporation. TAGs in lipoproteins are hydrolyzed by lipoprotein lipase (LPL), an enzyme localized on the surface of endothelial cells of many tissues, into NEFAs and 2-monoacylglycerols during circulation (Kurtovic et al., 2009). The resulting NEFAs are absorbed into each tissue via CD36, a fatty acid transporter (Abumrad et al., 1993), and used for β-oxidation or stored again as TAG after re-esterification. The remnant of the hydrolyzed VLDL is presumably formed into LDL (Sheridan, 1988). HDL is also the product from VLDL digested by LPL as revealed in isolated hepatocytes of Japanese eel (Yu et al., 1992). LDL and HDL mainly transport cholesterol and are eventually absorbed by liver and peripheral tissues via receptor-mediated endocytosis. The other way of TAG-rich lipoprotein incorporation is receptor-mediated endocytosis of plasma lipoproteins (Sakai et al., 1994). In mammalian studies, VLDL receptor is revealed to be involved in the CM and VLDL clearance in postprandial period (Goudriaan et al., 2004). Although there are few studies about lipoprotein receptors in fish, a previous study showed that the high fat line of rainbow trout has higher mRNA levels of VLDL receptor in its muscle (Kolditz et al., 2010), suggesting its contribution to lipids uptake into fish muscle.

*Torafugu, a typical lean fish species*

The total lipid contents of skeletal muscles in fatty fish vary depending on the dietary lipid level (Gélineau et al., 2001; Han et al., 2011; Lu et al., 2013). On the other hand, those in lean fish such as haddock *Melanogrammus aeglefinus* L., flounder, and torafugu showed constant levels irrespective of the dietary lipid level (Nanton et al., 2001; Lee and Kim, 2005; Kikuchi et al., 2009). Especially, torafugu marginally accumulates lipid in its skeletal muscle (approximately 1%), which does not show any changes depending on the seasons or nutritional conditions (Koizumi and Hiratsuka, 2009); meanwhile,

substantial lipids are deposited in its liver (approximately 65%). A previous study revealed that the gene expression level of LPL, an enzyme responsible for lipid uptake as described before, is significantly low in torafugu muscle compared to that in its liver (Kaneko et al., 2013). These reports suggest that the lipid accumulation in muscle is somehow repressed in torafugu and thus its liver would play an important role for maintaining lipid metabolism in torafugu whole body. Torafugu has the smallest genome among vertebrates and its whole-genome database is available (Aparicio et al., 2002), making it easy to undertake the experiments using gene information. Therefore, in this study, torafugu is chosen as a model lean fish species.

*Growth hormone signaling in fish*

Growth hormone (GH) is a peptide hormone produced and released from the pituitary gland and other sites in vertebrates. This hormone has pluripotent functions in regulating growth, osmoregulation, immunity, and energy metabolism. Especially, GH-transgenic and GH-treated fish show enhanced somatic growth (Fuentes et al., 2013), indicating that GH has significant effects in improving the growth rate of fish. To take advantage of the valuable effect of this hormone to aquaculture industry, it is meaningful to increase the knowledge of GH effects on fish metabolism from a broad perspective.

Many previous studies suggest that GH regulates the lipid metabolism in teleost species. GH-transgenic coho salmon *Oncorhynchus kisutch* has lower lipid content of whole body compared to non-transgenic individual (Raven et al., 2006). GH treatment decreases the lipid contents of liver and muscle in rainbow trout (Kling et al., 2012). These reports support the idea that fish GH has lipolytic effect observed in mammals (Sheridan, 1994). However, almost all studies were performed using fatty fish species as mentioned before. The present study therefore investigated the molecular function of GH on lipid metabolism in lean fish, torafugu, to understand the overall ability of GH in fish.

*The specific receptor for GH*

The GH signaling is transmitted through the specific receptor, GH receptor (GHR), on the cell surface of target tissues (Herrington and Carter-Su, 2001). GH binds to GHR and leads its dimerization with the conformation change that stimulates the tyrosine kinase activity of GHR. Subsequently, the dimerized GHR phosphorylates each other, resulting in the further activation of signaling molecules in cytoplasm including transcription factors to regulate target gene expressions. In mammal studies, skeletal muscle-specific GHR knockout mice show decreased activation of signaling molecules in skeletal muscle with peripheral adiposity (Mavalli et al., 2010). Liver-specific GHR knockout mice also display marked steatosis and decreased insulin-like growth factor I (IGF-I) gene expression, a primary target gene of GH in liver (Fan et al., 2009; List et al., 2014). Under the fasting condition, rainbow trout show decreased mRNA expressions of GHR and IGF-I in liver with reduced GH binding, although the circulating GH level is increased compared to those under the fed condition (Norbeck et al., 2007). These reports strongly indicate that the abundances of GHR in target tissues determine the GH responsiveness and physiological action.

*Ex vivo tissue culturing*

The energy metabolism is maintained in living body by numerous factors, such as endocrine and nervous systems. To observe the specific function of GH on each tissue, the effects derived from other factors should be eliminated. *Ex vivo* tissue culturing, keeping the tissue pieces in an artificial environment, is the optimal system to study such hormonal function. The results obtained from this system can be thought to reflect the behavior in a living body because of the maintained heterogeneous cell population of tissues. Many previous studies applied *ex vivo* system for investigating tissue functions in fish (O' Connor et al., 1993; Frolow and Milligan, 2004; Matsumoto et al., 2005). In this study, GH treatment was performed using *ex vivo* tissue culturing system for liver and muscle in serum-free

Leibovitz's L-15 medium, which is known as amino acid-rich medium and often used for fish cell culture.

*Transcriptome analyses*

With the previous progress of technologies for the gene expression analyses, the number of studies applying the transcriptome analyses for investigating fish metabolism has increased (Qian et al., 2014). As for torafugu studies, both DNA micoarray and RNA-seq technologies are revealed to be applicable (Matsumoto et al., 2014; Cui et al., 2014; Feroudj et al., 2014). DNA microarray is a hybridization-based technology using fluorescently labeled cDNA and oligonucleotide probes fixed onto a solid surface. RNA-seq, on the other hand, is a sequence-based technology determining the transcript sequences directly with the next generation sequencer. Both technologies have advantages and disadvantages, such as sensitivity, throughput, and cost per sample. In this study, transcriptome analysis using microarray was performed for comparing the effect of recombinant hormone to the same population of genes.

Based on these backgrounds, the present study investigated the molecular mechanisms of GH to maintain lipid metabolism in torafugu as outlined below.

In Chapter 1, the effects of torafugu GH on torafugu liver and muscle metabolism were investigated using *ex vivo* system. Torafugu GH cDNA was cloned and its open reading frame sequence was inserted into pQE-30 Xa bacterial vector. Using bacterial protein expression system, recombinant torafugu GH (rtGH) was produced. Tissue slices from liver and muscle of torafugu were cultured in L15 medium and treated with rtGH (5 ng/ml) for expected periods of time. Transcriptome analysis with oligo-microarray indicated that the effectc of GH on gene regulations were tissue-specific. The activation of GH signaling molecules was investigated by Western blot analysis using specific antibodies for signal transducer and activator of transcription (STAT), protein kinase B (Akt), and extracellular

signal-regulated kinase (ERK) to detect their phosphorylation ratio. As for liver slices, both Akt and ERK were significantly activated after 60 min stimulation with rtGH. On the other hand, in muscle slices, all of these signaling molecules tested in this study did not alter their activation during 60 min stimulation with rtGH. These results suggest that the sensitivity of rtGH signaling is also tissue-specific. Quantitative real-time PCR analysis for lipid metabolism related genes revealed that rtGH down-regulated the gene expressions of LPL and FAS after 1 and 12 hours of rtGH addition, respectively, and up-regulated those of CPT1 after 24 hours of rtGH addition in liver slices. These results indicate that rtGH repress the lipid influx and lipogenesis followed by promoting lipid consumption in liver. As for muscle slices, the gene expression of FAS was down-regulated after 12 hours of rtGH addition, suggesting the suppression effect of *de novo* fatty acid synthesis in muscle. Overall, these experiments indicate that rtGH has a function for regulating lipid metabolism in tissue-dependent manners.

In Chapter 2, cDNA cloning of torafugu growth hormone receptor isoforms was performed to understanding their molecular characteristics. The full-length cDNAs of two GHR isoforms, GHR1 and 2 were cloned and identified by phylogenetic analysis. The comparison of the deduced amino acid sequences of each GHR isoforms showed that torafugu GHR2 lacked two extracellular cysteine and five intracellular tyrosine residues, suggesting that these GHR isoform have different functions in GH/GHR signaling. The tissue distributions of the torafugu GHRs were investigated by quantitative real-time PCR using gill, heart, skin, fast muscle, slow muscle, liver, and intestine. As a result, all of these tested tissues were found to express both torafugu GHRs. The transcript abundance of torafugu GHR1 and GHR2 tended to be high in fast muscle, slow muscle, and liver. In mammal and other teleost fish, the highest relative mRNA levels of GHR have been found in the liver. In torafugu, notably, the significantly highest mRNA level of torafugu GHR1 was observed in fast muscle. Taking the results of Chapter 1 into account, these GHR distributions imply that the lipogenesis is strongly repressed in torafugu muscle by GH signaling.

The TAG distribution among tissues and serum lipoproteins would provide us with the information on lipid mobilization capacity. In Chapter 3, the difference of such distribution between torafugu and red seabream, a typical fatty fish species, were compared. As expected, torafugu accumulated TAG predominantly in liver and hardly did in muscle, whereas red seabream did in liver, muscle, and adipose tissue. However, the TAG contents in their sera were not so different from each other. Blue-native polyacrylamide gel electrophoresis followed by Oil red O staining also revealed that there was no significant difference in lipid distribution among lipoproteins. Further analysis using high-performance liquid chromatography demonstrated that TAG amount of a fraction containing VLDL and LDL was significantly higher in torafugu than red seabream (39.4 ± 15.6 vs. 24.7 ± 12.3 mg/100 mL, respectively), whereas those in the HDL fraction were similar (54.0 ± 23.1 vs. 51.4 ± 14.0 mg/100 mL, respectively). Since VLDL and LDL are thought to be major TAG transporters in fish, these results suggest that the low TAG content in torafugu muscle is not derived from the little TAG secretion from liver and intestine, but probably from low TAG incorporation into the muscle cell. This is also supported by further experiments using *ex vivo* system showing that the gene expression of 14 kDa apolipoprotein (Apo-14), a homologue of mammalian apolipoprotein A-II, was up-regulated by high concentration of rtGH (200 ng/ml) in liver slices. This result implies that torafugu is capable of producing the lipoproteins in its liver in response to GH stimulation.

Finally, the results obtained in this study were summarized and future perspectives were proposed in general discussion. The present study elucidated the molecular mechanisms of GH to maintain lipid metabolism in torafugu, possibly with controlling the lipid contents in liver and muscle. The knowledge obtained in this study will help us to understand the overall function and importance of GH in teleost fish species.

Some parts of this thesis have been already published as follows:

Hirano, Y., Kaneko, G., Koyama, H., Ushio, H., Watabe, S., 2011. cDNA cloning of two types of growth hormone receptor in torafugu *Takifugu rubripes*: tissue distribution is possibly correlated to lipid accumulation patterns. *Fisheries Science*. 77, 855–865

## Chapter 1

### Effects of recombinant torafugu growth hormone on liver and muscle tissue metabolism

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定。

## Chapter 2

### cDNA cloning and tissue distributions of two types of growth hormone receptor in torafugu

The biological action of GH is initiated by its binding to a specific receptor, the growth hormone receptor (GHR), located on the cell membrane of target tissues. After binding to GH, GHR is phosphorylated, resulting in activation of a cascade of signaling molecules, such as STAT, Akt, and ERK as mentioned in Chapter 1, that subsequently lead to the expression of target genes like transcription factors and metabolic enzymes. This entire process is called GH/GHR signaling (Herrington and Carter-Su, 2001).

GHR is a single transmembrane receptor protein belonging to the cytokine class I receptor superfamily. To date, cDNAs encoding GHR have been cloned from many kinds of vertebrates including various fish species (Reindl and Sheridan, 2012). Because of the genome duplication, almost all fish have at least two types of GHRs. One of the major functions of GH/GHR signaling is to up-regulate the expression of insulin-like growth factor-I (IGF-I), a peptide hormone having a critical role in somatic growth of vertebrates. IGF-I is involved in the regulation of metabolism as well as cell proliferation and differentiation, ultimately regulating body growth (Moriyama et al., 2000; Fuentes et al., 2013). This is called GH/IGF-I axis where GHR plays an important role. The previous studies using genetically modified mice revealed that the liver-specific deletion of GHR causes a 90% decrease in circulating IGF-I (Fan et al., 2009; List et al., 2014), indicating that the liver is the main target organ of GH to produce large quantities of IGF-I into circulation.

Several genes possibly related to growth rate in torafugu have been cloned including GH and IGF-I so far (Venkatesh and Brenner, 1997; Kaneko et al., 2011). Torafugu GHRs were also cloned *in silico* (Jiao et al., 2006; Kaneko et al., 2011), where the correlation of the mRNA expression levels of GH, GHR1, and IGF-I with growth rate has been insisted. Therefore, it is needed to confirm the actual

sequences of torafugu GHRs by cDNA cloning for further understanding of torafugu GH/GHR signaling. Interestingly, torafugu is typical lean fish, which accumulates lipids specifically in liver and hardly in muscle. GH has been reported to have direct lipolytic effects in fish liver (O' Connor et al., 1993; Bergan et al., 2013). It is also intriguing whether GH/GHR signaling involves such species-specific lipid accumulation patterns in fish.

In Chapter 2, cDNA cloning of torafugu GHRs were performed based on torafugu genome database. The comparison of deduced amino acid sequences with other vertebrates GHRs elucidated the characteristics of torafugu GHRs. The regulatory effects of GH on GHRs mRNA expressions in liver and muscle were also tested using *ex vivo* system. In addition, the capacity of torafugu liver to express the IGF-I gene responding to GH stimulation was demonstrated.

## Section 1. cDNA cloning and sequencing analysis of two types of GHR in torafugu

In this section, two types of torafugu GHRs were cloned *in silico* using torafugu whole-genome database with human GHR sequence as a probe. Gene-specific primers were designed based on the sequences obtained from toraufug genome database and the cDNAs including ORF were cloned. The deduced amino acid sequences were subsequently used for the primary structure and phylogenetic analyses.

### Materials and methods

*cDNA cloning of torafugu GHRs*

A specimen of torafugu reared in The University of Tokyo was used for cDNA cloning (n=1, approximately 65 g). Total RNAs were extracted from the fast muscle of torafugu using ISOGEN (Nippon Gene, Toyama, Japan) and treated with DNase I (TaKaRa) according to the manufacture's protocol. One μg of the total RNA was used as a template for the first-strand cDNA synthesis with Superscript III reverse transcriptase (Invitrogen) with an oligo(dT)20 primer (Invitrogen). Gene-specific primers GHR1F1, GHR1R1, GHR1F2, GHR1R2, GHR2F, and GHR2R were designed for cDNA cloning of GHR1 and GHR2 based on the JGI Fugu genome database version 4.0 (Table 2-1).

For GHR1, PCR was performed in a 50 μl reaction mixture containing 1 μl of first-strand cDNA, 5 μl of 10 x PCR buffer, 5 μl of 2 mM dNTP mixture, 3 μl of 25 mM $MgSO_4$, 20 pmol each of GHR1F1 and GHR1R1 primers, 13.2 μl of sterile distilled water, and 1 U of KOD-plus-ver.2 DNA polymerase (Toyobo). The PCR profile was as follows: 94˚C for 2 min, 30 cycles of 98˚C for 10 s, 57˚C for 45 s, and 68˚C for 2 min. The resulting products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and used as templates for nested PCR using GHR1F2 and GHR1R2 primers. For GHR2, PCR was performed in the same reaction mixture as above except for GHR2F and GHR2R

primers. The PCR profile was as follows: 94˚C for 2 min, 35 cycles of 98˚C for 10 s, 56˚C for 30 s, and 68˚C for 2 min.

*Rapid amplification of cDNA ends (RACE)*

To obtain the full sequence of the ORF of torafugu GHR2, 5' RACE was carried out. Total RNAs were extracted from various tissues of torafugu using RNeasy Lipid Tissue Mini Kit (Qiagen). First-strand cDNAs were synthesized from 1 μg of the total RNAs using Superscript III reverse transcriptase and a gene-specific primer, GSP-GHR2-RT (Table 2-1). The subsequent products were purified using MinElute Reaction Cleanup Kit (Qiagen) and incubated with 15 U of terminal deoxynucleotidyl transferase (TdT) and 2.5 μL of 2 mM dCTP (Invitrogen) at 37 ˚C for 20 min to add an oligo(dC) tail at the 5' end. PCR amplification was performed in 20 μl of a reaction mixture containing 1 μl of the TdT-treated cDNA, 2 μl of 10 x PCR buffer, 1.6 μl of 2.5 mM dNTP mixture, 10 pmol each of Abridged Anchor Primer (AAP) and GSP-GHR2-R1 primer (Table 2-1), 13.3 μl of sterile distilled water, and 0.5 U Ex Taq DNA polymerase (Takara). The PCR profile was as follows: 94˚C for 5 min, 25 cycles at 94˚C for 30 s, 54˚C for 30 s, 72˚C for 45 s, followed by a final extension step at 72˚C for 7 min. Subsequently the nested PCR was performed in 25 μl of a reaction mixture containing 1 μl of the PCR products, 2.5 μl of 10 x PCR buffer, 2.5 μl of 2 mM dNTP mixture, 1.5 μl of 25 mM $MgSO_4$, 10 pmol of Abridged Universal Amplification Primer (AUAP) and GSP-GHR2-R2 primer (Table 2-1), 6.6 μl of sterile distilled water, and 0.5 U of KOD-plus-neo DNA polymerase (Toyobo). The PCR profile was as follows: 94 ˚C for 2 min, 25 cycles of 98 ˚C for 10 s, 54 ˚C for 30 s, and 68 ˚C for 45 s.

3'RACE of torafugu GHR2 was also carried out. First-strand cDNAs were synthesized from 1 μg of the total RNAs by incubating with Superscript III reverse transcriptase and GeneRacer Oligo dT primer (Invitrogen) (Table 2-1). The following PCR was performed in 20 μl of a reaction mixture containing 1 μl of the cDNA, 2 μl of 10 x PCR buffer, 1.6 μl of 2.5 mM dNTP mixture, 10 pmol each of

GeneRacer 3'RACE primer (Invitrogen) and GSP-GHR2-F1 primer (Table 2-1), 13.3 μl of sterile distilled water, and 0.5 U Ex Taq DNA polymerase (Takara). The PCR profile was as follows: 94˚C for 5 min, 30 cycles at 94˚C for 30 s, 50˚C for 30 s, 72˚C for 2 min, followed by a final extension step at 72˚C for 7 min. The resulting products were used as templates for the nested PCR in 25 μl of a reaction mixture containing 1 μl of the products, 2.5 μl of 10 x PCR buffer, 2.5 μl of 2 mM dNTP mixture, 1.5 μl of 25 mM $MgSO_4$, 10 pmol of GeneRacer 3'RACE Nested primer (Invitrogen) and GSP-GHR2-F2 primer (Table 2-1), 6.6 μl of sterile distilled water, and 0.5 U of KOD-plus-neo DNA polymerase (Toyobo). The PCR profile was as follows: 94 ˚C for 2 min, 30 cycles of 98 ˚C for 10 s, 50 ˚C for 30 s, and 68 ˚C for 2 min.

*Sequence analysis*

The PCR products were ligated into pBluescript II KS (+) vector. The nucleotide sequences of purified plasmids were analyzed using a DNA sequencer ABI PRISM 3100 genetic analyzer. Subsequently the deduced amino acid sequences of GHR1 and GHR2 were determined. Signal peptides and transmembrane domains were predicted using SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM version 2.0 programs (http://www.cbs.dtu.dk/services/TMHMM/), respectively. N-glycosylation sites were predicted by using the software Gene Runner version 3.05 (Hasting Software, Hasting, USA). The exon-intron structures were determined with the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/) using the genomic sequences of the GHR1 gene (*GHR1*) and *GHR2* screened on the JGI Fugu genome database. The alignment of amino acid sequences was carried out by CLUSTAL W (Thompson et al., 1994). The phylogenetic tree of GHR was constructed using the maximum likelihood method on the MEGA v6.0 software with the substitution model JTT + G + I, which was determined by the evaluation of best-fit substitution (Tamura et al., 2013). Bootstrap resampling analysis from 1,000 replicates was used to

evaluate internal branches.

**Results**

*cDNA cloning of torafugu GHR1 and GHR2*

The BLAST program was run on the JGI Fugu genome database using the deduced amino acid sequence of human *Homo sapiens* GHR (NM000163) (Amselem et al., 1991) as a probe to identify torafugu counterparts. Two putative GHRs with high scores and low expect values were found in scaffolds 11 and 128. Based on the nucleotide sequences of these two putative torafugu GHRs, gene-specific primer pairs were designed to amplify their ORFs (Table 2-1). DNA fragments consisting of 1914 bp and 1590 bp in ORF were amplified by PCR using primer pairs designed based on the sequences in scaffolds 11 and 128, respectively. Further investigation using 5' RACE and 3'RACE revealed that the latter GHR had an additional 105 bp sequence containing 5'-untranslated region (UTR) and 5'-terminal ORF, and an additional 1024 bp sequence in 3'-UTR, yielding 1695 bp in the full-length ORF.

A phylogenetic tree was constructed by the maximum likelihood method based on the multiple alignment of deduced amino acid sequences of GHRs from torafugu, other fish, and several higher vertebrates, using the torafugu prolactin receptor (Lee et al., 2006) as the outgroup (Fig. 2-1). This analysis clearly showed that fish GHRs were clustered into two clades, namely GHR1 and GHR2. Torafugu GHRs in the longer and shorter ORF sequences were found to be categorized into GHR1 and GHR2, respectively. Fish GHR1 and GHR2 were both subsequently subdivided in two groups. One group was comprised of Perciformes and Pleuronectiformes, whereas the other of Siluriformes and Cypriniformes. Two GHR2 isoforms of Salmoniformes were monophyletic with the former group. Both torafugu GHRs also belonged to the former group.

The deduced amino acid sequence of torafugu GHR1 consisted of a signal peptide (23 aa), an

extracellular domain (234 aa), a single transmembrane domain (23 aa), and an intracellular domain (357 aa) (Fig. 2-2). That of torafugu GHR2 consisted of a signal peptide (23 aa), an extracellular domain (221 aa), a single transmembrane domain (23 aa), and an intracellular domain (297 aa) (Fig. 2-2). The multiple alignment of torafugu and other vertebrate GHRs revealed that torafugu GHR1 had several common features including a signal peptide, seven conserved extracellular cysteine residues, potential *N*-glycosylation sites, a GHR ligand-binding motif of FGEFS, nine conserved intracellular tyrosine residues, and box 1 and box 2 domains (Fig. 2-2). As for torafugu GHR2, it contained only five conserved extracellular cysteine residues and four conserved intracellular tyrosine residues.

The results from the phylogenetic analysis were consistent with the amino acid sequences identities of torafugu GHR1 and GHR2 with GHRs from other vertebrates. Torafugu GHR1 and GHR2 always showed higher identity to fish ones than to other animal species GHRs including amphibians and mammals when compared using the mature receptor sequences or using extracellular and intracellular domains separately (Table 2-2 and 2-3). The approximate identities of torafugu GHR1 mature receptor with Perciformes, Pleuronectiformes, Siluriformes, and Cypriniformes were 70, 65, 40, and 50%, respectively, whereas those of torafugu GHR2 mature receptor with Periciformes, Salmoniformes, Siluriformes, and Cypriniformes were 60, 50, 40, and 40%, respectively. Meanwhile, those of torafugu GHR1 and GHR2 mature receptors with other animal species GHRs were only about 30%, which were lower than those with fish. As for GHR1, orange-spotted grouper counterpart showed the highest amino acid identity to torafugu one (70%), whereas the value between torafugu and orange-spotted grouper GHR2s was only 61%, suggesting that torafugu GHR1 is more conserved than torafugu GHR2.

Table 2-4 shows the amino acid sequence identities between the two GHR isoforms of each fish species in the mature form, extracellular domains, and intracellular domains. The amino acids identity of the extracellular domain was found to be higher than that of the intracellular domain in any fish compared irrespective of GHR1 and GHR2, indicating that the extracellular domain is more likely to

be conserved than the intracellular domain. Among these six species, the identity between the isoforms in intracellular domain was various (from lowest 28% in torafugu and zebrafish to highest 41% in orange-spotted grouper), although the identity in the extracellular domain was similar (41, 46 and 45% in torafugu, zebrafish and orange-spotted grouper, respectively). Torafugu GHR isoforms in the mature form showed the lowest identity between each other among six fish species compared in this study.

Genomic organizations of torafugu GHR1 and GHR2 were revealed based on the Fugu genome database together with the cDNA sequences obtained in the present study (Fig. 2-3). The information of genomic DNA sequence is also available on Ensemble database with ensemble IDs ENSTRUG00000015040 and ENSTRUG00000013005 for scaffold 11 and 128, respectively. The genomic DNA of torafugu GHR1 was composed of nine exons, where exon 1 coded for the signal peptide, exons 2-5 for the extracellular domain, exon 6 for the transmembrane domain, and exons 7-9 for the intracellular domain. As for torafugu GHR2, the genomic DNA was composed of eight exons, where exons coded for the corresponding domains as GHR1 except that the intracellular domain of GHR2 was encoded by exon 7-8. There were the largest intron sizes between exons 1 and 2 in both torafugu GHRs genomic DNAs. Comparison of the genomic structure of GHRs between torafugu and zebrafish elucidated that the corresponding translated regions between torafugu and zebrafish GHRs are encoded by the same exons.

## Section 2. Tissue distributions of torafugu GHR1 and GHR2

As mentioned in General introduction, the abundance of GHR would determine the GH responsiveness of each tissue. In this section, the mRNA expression levels of torafugu GHR1 and GHR2 in various tissues were measured by quantitative real-time PCR to estimate their GH sensitivity.

### Materials and methods

*Fish*

Torafugu whose tissues were used for reverse transcription (RT)-PCR followed by quantitative real-time PCR (n=4, average body weight 1,100 g) were obtained from a commercial dealer (Fish Interior). Before sampling, they were reared in a 1-t tank for 1 day in The University of Tokyo. After anesthesia with ice-cold seawater for approximately 15 min, various tissues were dissected and stored in RNA later at -80 ˚C until RNA extraction. Samples dissected from dorsal white and erector depressor slow muscles at the base of the dorsal fin were used as fast muscle and slow muscle, respectively.

*Quantitative real-time PCR for tissue distributions of GHR1 and GHR2*

The relative mRNA levels of GHR1 and GHR2 were analyzed by quantitative real-time PCR with an ABI PRISM 7300 real-time PCR system. Those of torafugu β-actin were used as the internal control. The gene-specific primer pairs were used for GHR1 (GHR1 real-time F and GHR1 real-time R) and β-actin (β-actin real-time F and β-actin real-time R) (Kaneko et al., 2011), whereas those for GHR2 (GHR2 real-time F and GHR2 real-time R) were designed using the Primer Express software version 2.0 (Table 2-1). The quantitative PCR was performed in a 20 μl reaction mixture that contained 2 μl of cDNA (1:10 dilution), 0.4 μl of 50 x ROX Reference Dye (Takara), 10 pmol each of gene-specific primers, and

10 μl of 2 x SYBR Premix Ex Taq (Takara). The PCR profile was as follows: 95˚C for 30 s, 40 cycles at 95˚C for 5 s and 60˚C for 31 s. The absence of non-specific PCR products was verified by running the dissociation protocol (95˚C for 15 s, 60˚C for 30 s, and 95˚C for 15 s). The relative mRNA levels were calculated by the comparative Ct method with reference to those of β-actin as the internal control. The resulting data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post-hoc* test.

**Results**

*Tissue distribution of torafugu GHR1 and GHR2*

The reverse transcribed cDNAs from various tissues including gill, heart, skin, fast muscle, slow muscle, liver, and intestine were used for RT-PCR followed by quantitative real-time PCR. The transcripts of torafugu GHR1 and GHR2 were obtained by both RT-PCR and quantitative real-time PCR in all tissues examined (data not shown for RT-PCR). In detail results of the relative mRNA levels of torafugu GHR1 and GHR2 obtained by quantitative real-time PCR was shown in Fig. 2-4. Statistically significant difference in the relative mRNA levels of GHR1 and GHR2 among these tissues was shown by One-way ANOVA ($p < 0.05$). Subsequent Tukey-Kramer HSD *post-hoc* test revealed that fast muscle accumulated significantly highest relative mRNA level of GHR1 than those in other tissues ($p < 0.05$). On the other hand, no significant difference was observed in the relative mRNA level of GHR2 among all tissues examined.

## Section 3. The effects of rtGH on torafugu GHRs and IGF-I gene expressions

Some previous reports showed that GH regulates GHR expression in fish hepatocytes (Very and Sheridan, 2007; Pierce et al., 2012). In this section, the rtGH potency to regulate the gene expressions of GHR1 and GHR2 in torafugu liver and muscle slices was evaluated. As for liver, many fish species are reported to up-regulate IGF-I gene expression responding to GH stimulation (Pierce et al., 2004; Leung et al., 2008; Pedroso et al., 2009; Pierce et al., 2011; Reindl et al., 2011). To reveal whether torafugu liver also shows the similar response to GH, the IGF-I gene expressions in liver slices treated with different rtGH concentrations were confirmed.

### Materials and methods

*Preparation of recombinant torafugu GH and liver slices for tissue culturing*

The detailed methods for the preparation of rtGH and tissue culturing of liver and muscle were described in Chapter 1. The purified rtGH dissolved in hyperosmolar PBS were added to culture wells to achieve the desired final concentrations (5 ng/ml and 200 ng/ml). Control cultures received an equivalent volume of hyperosmolar PBS. The liver samples were then collected after 12 and 24 hours of rtGH addition. The slices were immersed in RNA later and stored at -20˚C.

*Quantitative real-time PCR for tissue culture experiments*

The total RNAs were extracted from tissue slices and reverse transcribed as described in Chapter 1. For each gene, the gene-specific primers and TaqMan probes were designed using Primer Express software version 2.0 as listed in Table 2-1. The enzyme and profile used for real-time PCR was the same as those used for evaluating the gene expressions in Chapter 1. Relative mRNA levels were determined by the comparative Ct method with reference to those of EF-1$\alpha$ as the internal control.

*Statistical analysis*

Data are expressed as means ± SE. Statistical differences of the relative mRNA levels between control and rtGH treatment groups were determined by Student's *t*-test or Welch's *t*-test after F-test for two means.

**Results**

*The effects of rtGH on the gene expressions of GHR1 and GHR2 in torafugu liver and muscle*

The quantitative real-time PCR for GHR1 and GHR2 was performed using the liver and muscle slices treated with rtGH at final concentration of 5 ng/ml. The gene expression changes were evaluated after 12 and 24 hours of rtGH addition (Fig. 2-5). There was no significant change in the gene expressions of both GHR1 and GHR2 irrespective of the tissue slices by rtGH treatment.

*The effects of rtGH on the gene expressions of IGF-I in torafugu liver*

The relative mRNA levels of IGF-I in the liver slices treated with rtGH at two different concentrations (5 and 200 ng/ml) were measured by quantitative real-time PCR. The gene expression change was evaluated after 12 and 24 hours of rtGH addition (Fig. 2-6). At both concentrations of rtGH treatment, there was no significant change in the gene expression of IGF-I in liver slices.

## Section 4. Discussion

There have been many reports identifying the two distinct GHRs in teleosts (Reindl and Sheridan, 2012). In this study, the two types of cDNAs coding torafugu GHRs were cloned, both of which had conserved regions in extracellular and intracellular domains. In the extracellular domain, the ligand-binding motif (FGEFS) was found as in the cases of frog, chicken and almost all fish species reported so far (Fig. 2-2). It is noted that this motif is substituted to YGEFS in human, YSGFS in mouse, and EGGFS in turtle. In the intracellular domain, the sequences corresponding to box 1 and box 2 were found in torafugu GHRs. Box1 is revealed as a site for JAK2 binding in human GHR, whereas box 2 is involved in the proliferative response (Frank et al., 1994; Ihle et al., 1995). The observation that those regions were highly conserved in torafugu GHRs suggests that a signaling pathway downstream of GHRs similarly function in torafugu as in other animals.

Torafugu GHR1 had seven conserved extracellular cysteine residues (Fig. 2-2). When compared with human GHR, the three pairs of these extracellular cysteine residues, C52/C62, C97/C107, and C121/C137 are expected to form disulfide bonds. Human GHR has been revealed to dimerize via binding to GH and form an intermolecular disulfide bond between the unpaired cysteine residues of two GHR molecules (Zhang et al., 1999). It remains to be elucidated whether torafugu GHR counterpart, C229, the last unpaired cysteine residues, also participates in dimerization. Meanwhile, six potential *N*-glycosylation sites were found in the extracellular domain of torafugu GHR1. The involvement of GHR glycosylation in an affinity efficiency of GHR to GH and in GH-GHR complex internalization is claimed in a previous study (Harding et al., 1994). There were nine tyrosine residues in the intracellular domain in torafugu GHR1. Such tyrosine residues in the intracellular domain are probably phosphorylated after dimerization and subsequently activate the downstream signaling molecules based on the mammalian GHR investigations (Wang et al., 1996; Hansen et al., 1997).

On the other hand, torafugu GHR2 lacked two extracellular cysteine residues compared with torafugu GHR1 (Fig. 2-2): the cysteine residues corresponding to C121 and C137 in GHR1 were not observed in GHR2. It is expected that torafugu GHR2 has less numbers of intramolecular disulfide bonds than GHR1. Torafugu GHR2 also lacked three *N*-glycosylation sites and five intracellular tyrosine residues compared with torafugu GHR1. The less numbers of these residues in torafugu GHR2 might cause less efficient function in GH/GHR signaling. Actually, a previous report investigating black seabream *Acanthopagrus schlegeli* showed that the receptor-mediated promoter activation by GHR2 was much lower than that of GHR1 that were over-expressed in cultured Chinese hamster ovary cells (Jiao et al., 2006). The amino acid identities between GHR1 and GHR2 in the same fish species revealed that torafugu GHRs were less similar than black seabream GHRs (Table 2-4), suggesting that torafugu GHR1 and GHR2 also have different functions in GH/GHR signaling.

Torafugu GHRs identified in this study were clearly clustered into different clades, fish GHR1 and GHR2 (Fig. 2-1). Torafugu belongs to Tetraodonitiformes and its two GHRs were divided into those from Perciformes and Pleuronectiformes, indicating the evolutionarily close relationship among these species than the other three groups including Siluriformes, Cypriniformes, and Salmoniformes. The amino acid identities between GHR1 and GHR2 in the same fish species showed that the identity of the extracellular domain of GHRs was higher than that of the intracellular domain in six fish species examined (Table 2-4), indicating that the extracellular domain is more conserved than the intracellular domain evolutionarily. The exon-intron organization was remarkably similar between torafugu GHR1 and GHR2 (Fig. 2-3), where the regions encoding the signal peptide and the transmembrane domain were located at exon 1 and 6, respectively. This organization was almost the same as in zebrafish GHRs (Prinzio et al., 2010), suggesting the highly conserved genomic organizations of GHRs in fish.

The mRNA expressions of both torafugu GHR1 and GHR2 were observed in all tissues examined at different levels (Fig. 2-4). The higher relative mRNA levels of both transcripts were detected

in fast muscle, slow muscle, and liver. Especially, the significantly highest relative mRNA level of torafugu GHR1 was observed in fast muscle. The expression level of GHR is generally higher in liver than that in muscle in various fish species (Tse et al., 2003; Jiao et al., 2006; Small et al., 2006; Fuentes et al., 2008; Walock et al., 2013). In this regard, it is interesting that the relative mRNA level of GHR1 in muscle was higher than that in liver from torafugu. A previous study using gene modified mice showed that the liver-specific GHR deficiency causes hepatic steatosis, implying that the remarkable lipid content in torafugu liver is derived from the relatively lower GHR expression in torafugu liver. The *ex vivo* experiments revealed that the gene expression of GHR1 and GHR2 in liver and muscle slices were not significantly changed by rtGH (Fig. 2-5). Actually, the previous studies investigated the effect of GH on GHR expression in several fish species, but the results are conflicting. The GHR1 and GHR2 expressions in isolated rainbow trout hepatocytes are significantly up-regulated by trout GH (Very and Sheridan, 2007), whereas only the expressions of GHR2 is strongly stimulated by native tilapia GH in primary cultured tilapia hepatocytes (Pierce et al., 2012). The *in vivo* GH treatment in black seabream does not show any change in the gene expression levels of GHR1 and GHR2 (Jiao et al., 2006). Taking these reports into account, the results obtained in this study suggest that the effect of GH on GHR expression is species-specific and that the highest expression of GHR1 in torafugu muscle is possibly regulated by other factors, such as hormones other than GH and/or sex steroids (Reindl et al., 2012). It is required to identify the main factor which maintaining the GH/GHR signaling in torafugu whole body.

The *ex vivo* experiments also revealed that the gene expression of IGF-I in torafugu liver slices did not changed by rtGH treatment at both low and high concentration (5 and 200 ng/ml, respectively) (Fig. 2-6). In contrast to the GHR expression, the results obtained from the previous studies investigating the GH effect on IGF-I expression in fish liver are consistent with each other. Namely, the GH treatment significantly up-regulates the IGF-I expression in teleost hepatocytes (Pierce et al., 2004; Leung et al., 2008; Pedroso et al., 2009; Pierce et al., 2011; Reindl et al., 2011). In this study, the IGF-I

gene expression was not regulated by rtGH treatment in torafugu liver slices although its concentration used in this study was comparable to the previous studies. This result suggests that the torafugu liver has low GH sensitivity with regard to IGF-I production compared to other fish species, possibly reflecting the relatively lower expression of GHRs. The tissue distribution of IGF-I in torafugu tissues was investigated only by reverse transcribed PCR (Kaneko et al., 2011). The detailed information of IGF-I tissue distribution with quantitative real-time PCR will provide us the correlation between GHR and IGF-I gene expressions in various tissues.

In conclusion, two kinds of GHR, GHR1 and GHR2, were cloned from torafugu in this study. The amino acid sequences of functionally important regions were highly conserved with other vertebrates in both GHR1 and GHR2. As for GHR1, the low relative mRNA level in liver compared to that in skeletal muscle was observed by quantitative real-time PCR. The *ex vivo* experiments showed that the rtGH did not regulate the gene expression of IGF-I in torafugu liver slices. These results suggest that torafugu has low GH sensitivity in liver, probably relating to its high lipid content.

**Table 2-1.** Sequences of primers used in cDNA cloning, RT-PCR and quantitative real-time PCR

| Primer name | Sequence (5' - 3') | Gene | Tissue |
|---|---|---|---|
| *For cDNA cloning* | | | |
| GHR1F1 | GCTCTGATTCTCTCCGCAGCATC | *GHR1* | - |
| GHR1R1 | CTGAAGGTCTAAGGCTAGTGTCAC | *GHR1* | - |
| GHR1F2 | CGCAGCATCATGGCTGTCTCC | *GHR1* | - |
| GHR1R2 | GTCACTTTAACGGGACAGGTTCC | *GHR1* | - |
| GHR2F | GGTCCGTCTCCACACATCACC | *GHR2* | - |
| GHR2R | CTATGGGGTGATGCTTCCCAAAAGATCAGG | *GHR2* | - |
| *For 5' RACE* | | | |
| GSP-GHR2-RT | TCCTCGGCCCAGGAACAGGTGGCAAAAG | *GHR2* | - |
| GSP-GHR2-R1 | GTCAGCTCCTGCGGAGGGTCTGGTTGTA | *GHR2* | - |
| GSP-GHR2-R2 | ATGGTGTAAATGTCCAGACG | *GHR2* | - |
| AAP | GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG | - | - |
| AUAP | GGCCACGCGTCGACTAGTAC | - | - |
| *For 3' RACE* | | | |
| GeneRacer Oligo dT | GCTGTCAACGATACGCTACGTAACGGCATGACAGT G(T)24 | *GHR2* | - |
| GeneRacer 3'RACE | GCTGTCAACGATACGCTACGTAACG | *GHR2* | - |
| GSP-GHR2-F1 | GAGTCCGCTGCAAAACTCTC | *GHR2* | - |
| GeneRacer 3'RACE Nested | CGCTACGTAACGGCATGACAGTG | *GHR2* | - |
| GSP-GHR2-F2 | TCATGCAACCCTCACTCTTG | *GHR2* | - |
| *For quantitative real-time PCR for tissue distributions* | | | |
| GHR1 real-time F | AGAGTACGAGGTGCACATTCGTT | *GHR1* | - |
| GHR1 real-time R | AGTCGCTGAATTCTCCAAACTTTT | *GHR1* | - |
| GHR2 real-time F | GCAGGAAAAGTTGATGGTTCTTCTT | *GHR2* | - |
| GHR2 real-time R | TTTGAGTCGACCCCTCTTATCC | *GHR2* | - |
| β-actin real-time F | CGCCGCACTCGTTGTTG | *b-actin* | - |
| β-actin real-time R | CTCCAGCAAATCCGGCTTT | *b-actin* | - |
| *For quantitative real-time PCR of tissue culture samples* | | | |
| GHR1 real-time F1 | ATTGAAGTGACTGAAATTCCCATC | *GHR1* | L/- |
| GHR1 real-time F2 | TGAAGTGACTGAAATTCCCATCA | *GHR1* | -/M |
| GHR1 real-time R1 | ATACTGACGGCCCCAAACAC | *GHR1* | L/- |
| GHR1 real-time R2 | TACTGACGGCCCCAAACAC | *GHR1* | -/M |
| GHR1 real-time probe | AGTCTCCCTTCTCTCTCACCCTGGCACT | *GHR1* | L/M |
| GHR2 real-time F1 | GCAGCAAAAGTTGATGGTTCTTCTT | *GHR2* | L/- |
| GHR2 real-time F2 | TTGTCCACATCCCGTCCAA | *GHR2* | -/M |
| GHR2 real-time R | CAATTTTGAGTCGACCCCTCTT | *GHR2* | L/M |
| GHR2 real-time probe | TGCCACCTGTTCCTGGGCCG | *GHR2* | L/M |
| IGFI real-time F | GGAAGTGCATCAGAAAAACTCAAG | *IGF-I* | L/- |
| IGFI real-time R | ATTCGCTCCTCGCCTACATTC | *IGF-I* | L/- |
| IGFI real-time probe | TCGAGGCAATGCGGGTGGC | *IGF-I* | L/- |
| qRT-EF-1a-F1 | CGTTGTTGGCGACAGCAA | *EF-1a* | L/M |
| qRT-EF-1a-R1 | TGATGACCTGGGCATTGAAG | *EF-1a* | L/M |
| qRT-EF-1a-R2 | ATGATGACCTGGGCATTGAAG | *EF-1a* | L/- |
| qRT-EF-1a-probe | AACGACCCACCAAAGGGAGCTGACA | *EF-1a* | L/M |

AAP, Abridged Anchor Primer; AUAP, Abridged Universal Amplification Primer. L, primes and probes used for liver samples; M. primers and probes used for muscle samples.

**Table 2-2.** Amino acid sequence identities of torafugu GHR1 with those from other fish and torafugu GHR2 and PRLR

| Species | Accession No. | Amino acid identity | | |
|---|---|---|---|---|
| | | Mature form | Extracellular domain | Intracellular domain |
| GHR1 | | | | |
| Orange-spotted grouper | EF052273 | 70 | 78 | 66 |
| Gilthead seabream | AF438176 | 69 | 77 | 65 |
| Black seabream | AF502071 | 69 | 78 | 65 |
| Turbot | AF352396 | 66 | 75 | 61 |
| Japanese flounder | AB058418 | 65 | 73 | 63 |
| Nile tilapia | AY973232 | 64 | 73 | 59 |
| Goldfish | AF293417 | 48 | 55 | 45 |
| Rohu | AY691177 | 49 | 53 | 47 |
| Catla | AY691178 | 49 | 53 | 47 |
| Catfish | AY336104 | 42 | 52 | 39 |
| Zebrafish | EU649774 | 42 | 54 | 38 |
| GHR | | | | |
| Frog | AF193799 | 32 | 41 | 27 |
| Turtle | AF211173 | 33 | 41 | 30 |
| Chicken | NM001001293 | 33 | 39 | 30 |
| Rat | NM017094 | 31 | 38 | 27 |
| Mouse | NM010284 | 32 | 38 | 28 |
| Rabbit | NM001082636 | 31 | 38 | 28 |
| Cow | NM176608 | 31 | 39 | 27 |
| Sheep | NM001009323 | 32 | 40 | 27 |
| Human | NM000163 | 31 | 35 | 28 |
| Others | | | | |
| Torafugu PRLR | NP001072093 | 22 | 28 | 16 |
| Torafugu GHR2 | AB621337 | 34 | 41 | 28 |

**Table 2-3.** Amino acid sequence identities of torafugu GHR2 with those from other fish and torafugu GHR1 and PRLR

| Species | Accession No. | Amino acid identity | | |
|---|---|---|---|---|
| | | Mature form | Extracellular domain | Intracellular domain |
| GHR2 | | | | |
| Orange-spotted grouper | EF052274 | 61 | 63 | 58 |
| Gilthead seabream | AY573601 | 60 | 61 | 57 |
| Black seabream | AF662334 | 59 | 61 | 57 |
| Nile tilapia | AY973233 | 55 | 55 | 53 |
| Coho salmon isoform 1 | AF403539 | 48 | 53 | 44 |
| Coho salmon isoform 2 | AF403540 | 49 | 51 | 47 |
| Catfish | AY97323 | 41 | 43 | 38 |
| Zebrafish | EU649775 | 38 | 43 | 34 |
| GHR | | | | |
| Frog | AF193799 | 30 | 35 | 28 |
| Turtle | AF211173 | 31 | 36 | 28 |
| Chicken | NM001001293 | 30 | 34 | 28 |
| Rat | NM017094 | 29 | 35 | 29 |
| Mouse | NM010284 | 31 | 33 | 29 |
| Rabbit | NM001082636 | 31 | 36 | 29 |
| Cow | NM176608 | 33 | 37 | 30 |
| Sheep | NM001009323 | 33 | 36 | 30 |
| Human | NM000163 | 31 | 36 | 28 |
| Others | | | | |
| Torafugu PRLR | NP001072093 | 23 | 29 | 19 |
| Torafugu GHR1 | AB621336 | 34 | 41 | 28 |

**Table 2-4.** Amino acid sequence identities between GHR1 and GHR2 in fish

| Species | Mature form | Extracellular domain | Intracellular domain |
|---|---|---|---|
| Orange-spotted grouper | 43 | 45 | 41 |
| Gilthead seabream | 42 | 46 | 39 |
| Black seabream | 41 | 46 | 38 |
| Nile tilapia | 38 | 44 | 33 |
| Zebrafish | 36 | 46 | 28 |
| Torafugu | 34 | 41 | 28 |

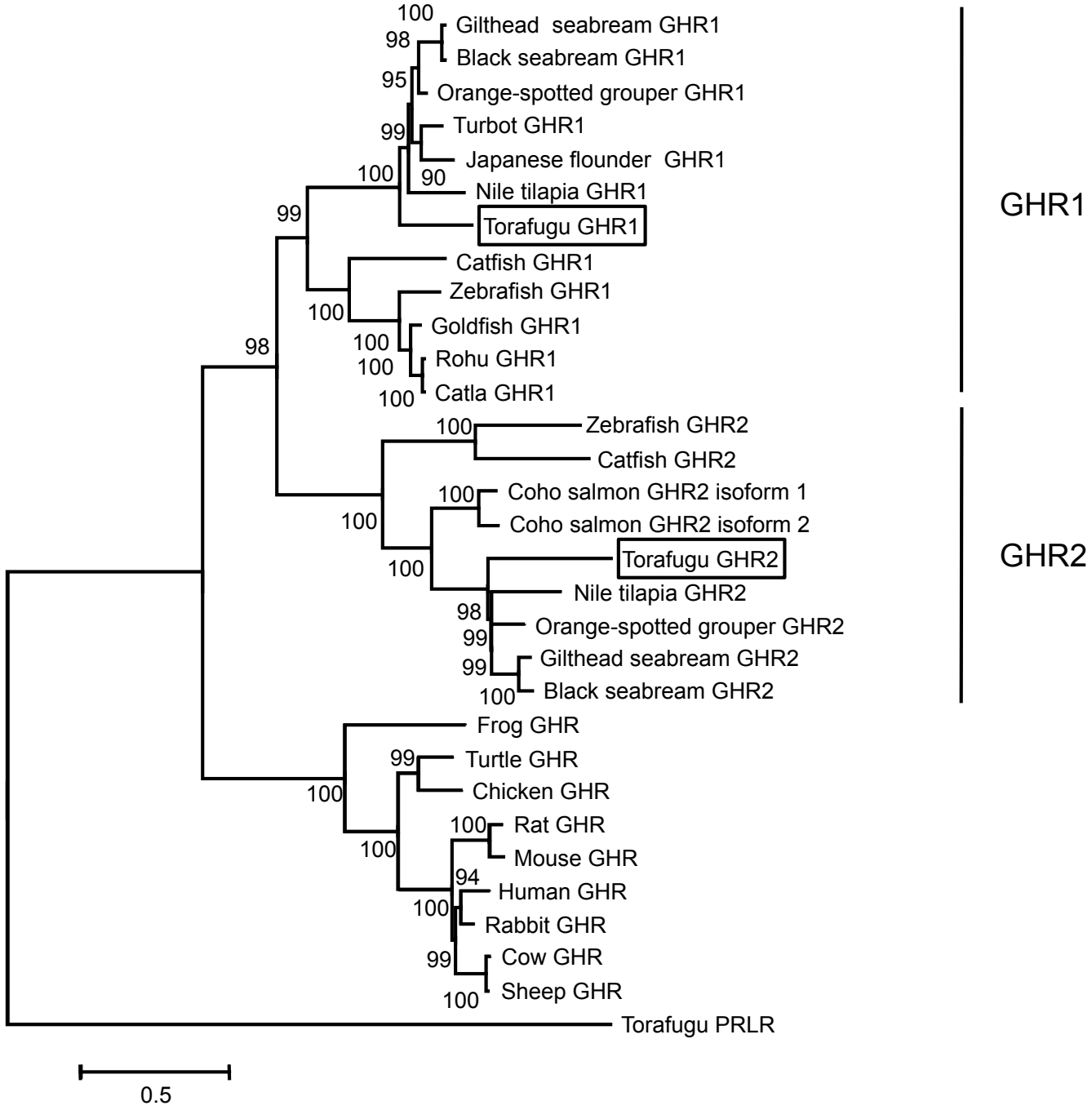


**Fig. 2-1.** The phylogenetic tree constructed by MEGA v6.0 using the maximum likelihood method based on the deduced amino acid sequences of GHRs from vertebrate animals. The bootstrap values from a 1,000-replicate analysis are given at the nodes in percentage. The scale bar, phylogenetic distance of 0.5 amino acid substitution per site. The accession numbers of proteins in the NCBI database are shown in Table 2-2 and 2-3.

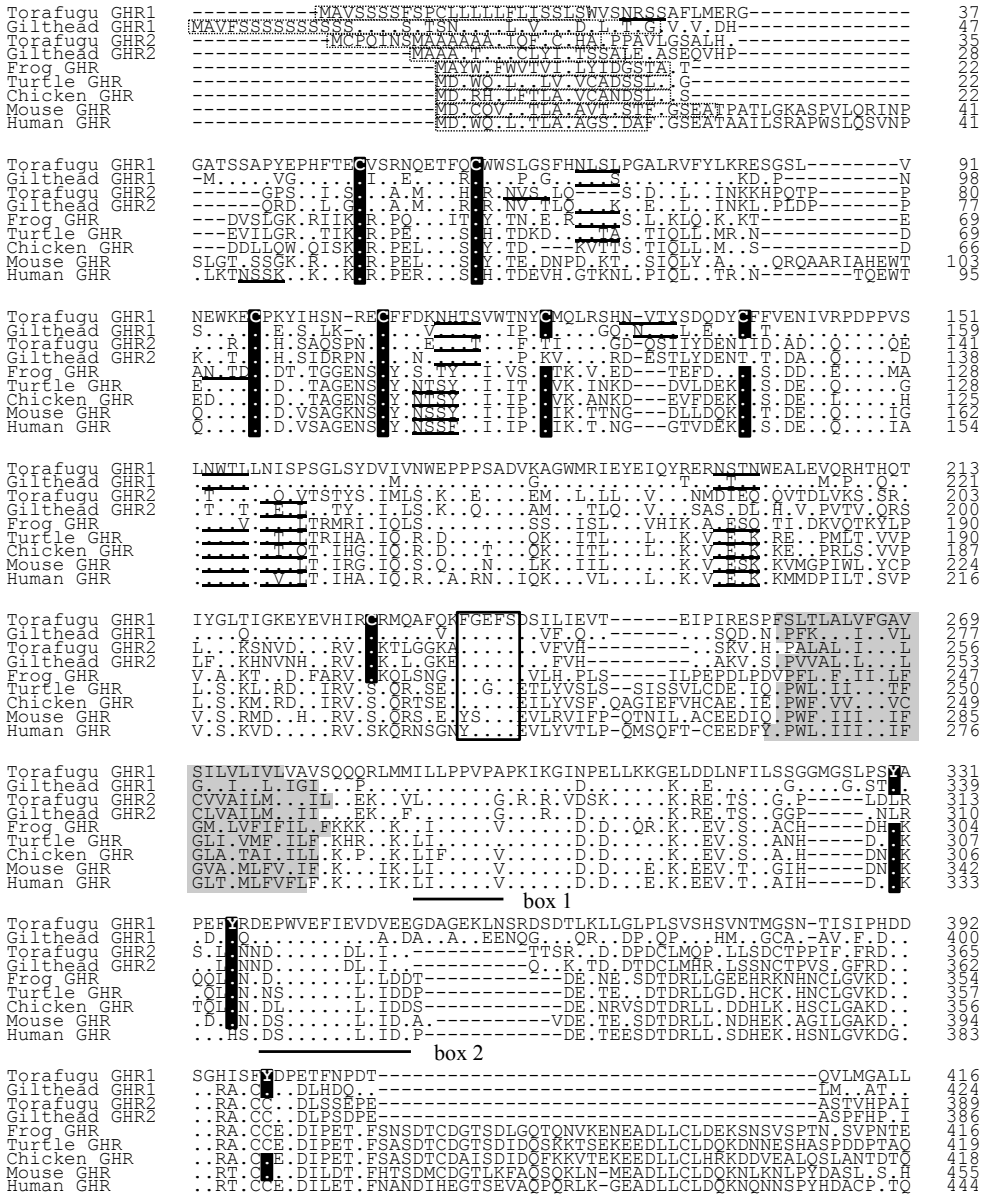


**Fig. 2-2.** The alignment of the deduced amino acid sequences of torafugu GHR1 and GHR2 with those of GHRs from other vertebrate animals. Amino acid residues identical to those of torafugu GHR1 are indicated with dots. Dashes denote gaps to optimize the alignment. Numbers on the right margin show those from the N-terminal methionine. Putative signal peptides are boxed with dotted-line. Potential *N*-glycosylation sites are underlined. Conserved cysteine residues in the extracellular domain and tyrosine residues in the intracellular domain are highlighted in dark. Ligand binding motifs are represented by an open box. The transmembrane domains is shaded. Conserved boxes 1 and 2 are underlined and described as so. The accession numbers of proteins in the NCBI database are shown in Tables 2 and 3

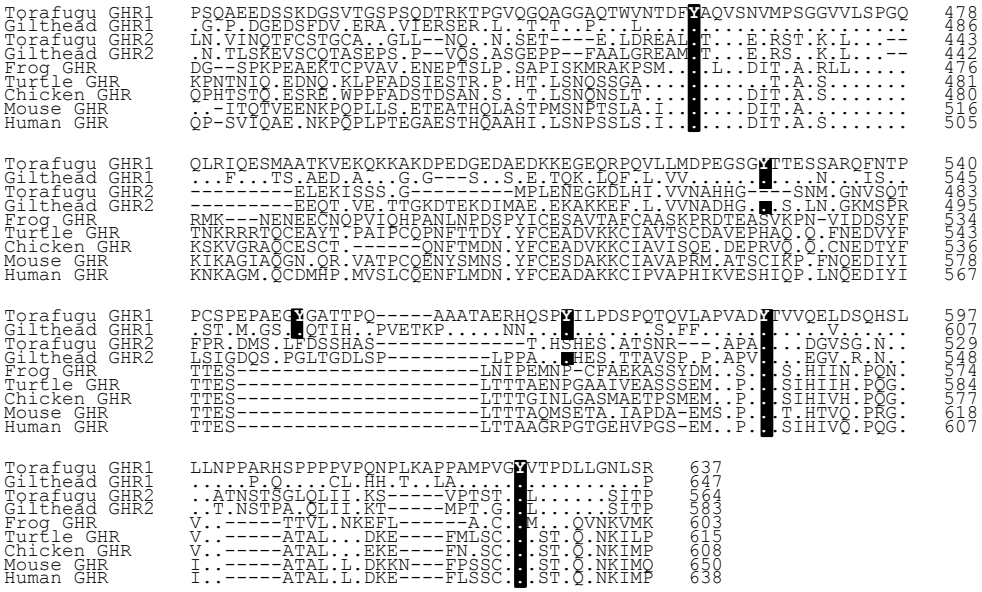

**Fig. 2-2.** Continued.

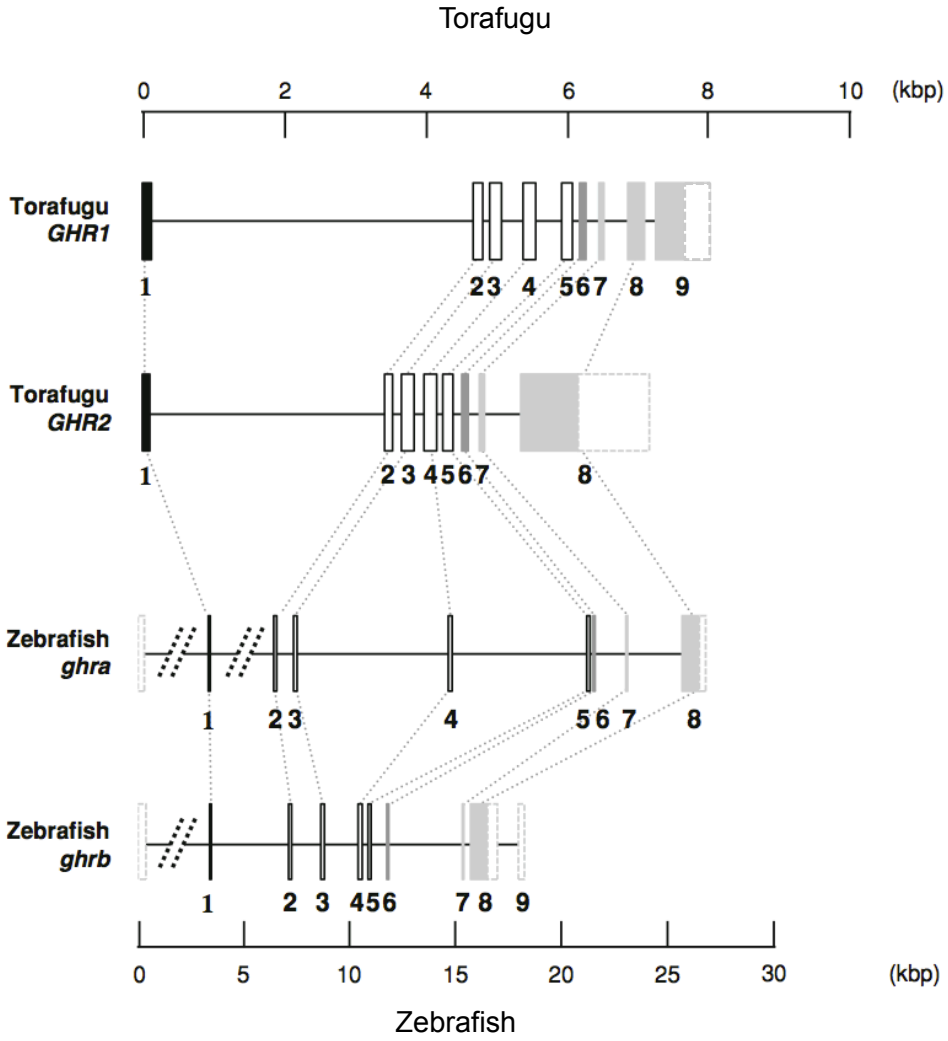


**Fig. 2-3.** Comparison of the exon-intron structures of *GHRs* between torafugu and zebrafish. Exons are shown by boxes, whereas introns by solid lines. Exons encoding signal peptide, extracellular domain, transmembrane domain, and intracellular domain are represented with black, white, dark gray, and light gray boxes, respectively. Untranslated regions are indicated with dotted-line boxes. Dotted lines connect corresponding exons among all structures. Torafugu *GHR1* and *GHR2* exons and zebrafish counterparts are numbered from the exon including the start codon. First exons of torafugu *GHR1* and *GHR2*, zebrafish *ghra* and *ghrb* include 9, 11, 16 and 10 bp of 5'-UTR, respectively

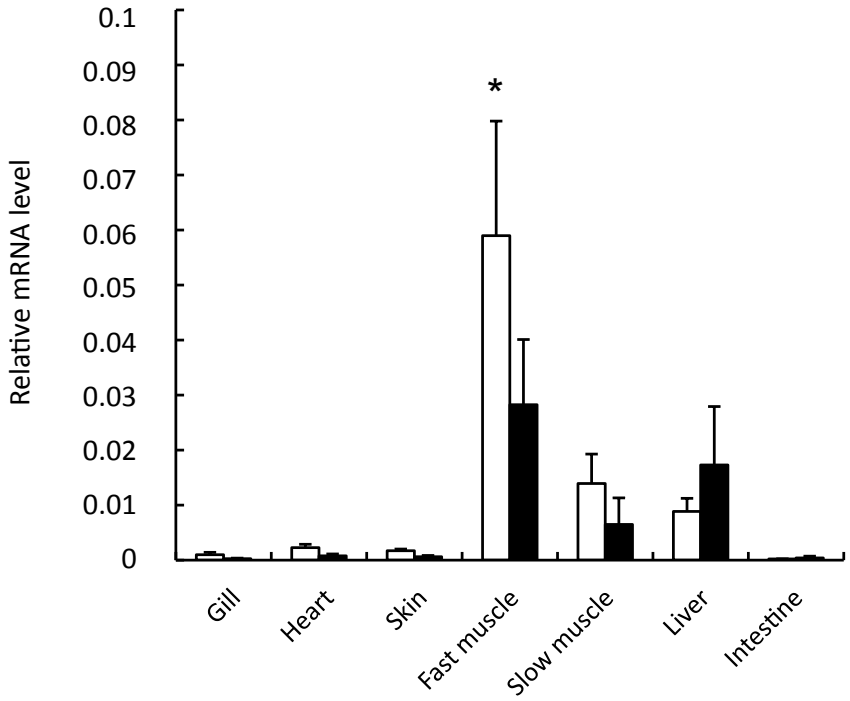


**Fig. 2-4.** The relative mRNA levels of torafugu GHR1 and GHR2 in various tissues. The relative mRNA levels of GHR1 (□) and GHR2 (■) were calculated using those of the β-actin gene as the internal control. Bars represent means ± SE ($n$=4). Significant difference ($p < 0.05$) in the relative mRNA levels of GHR1 and GHR2 among tissues examined is shown by asterisk (*)

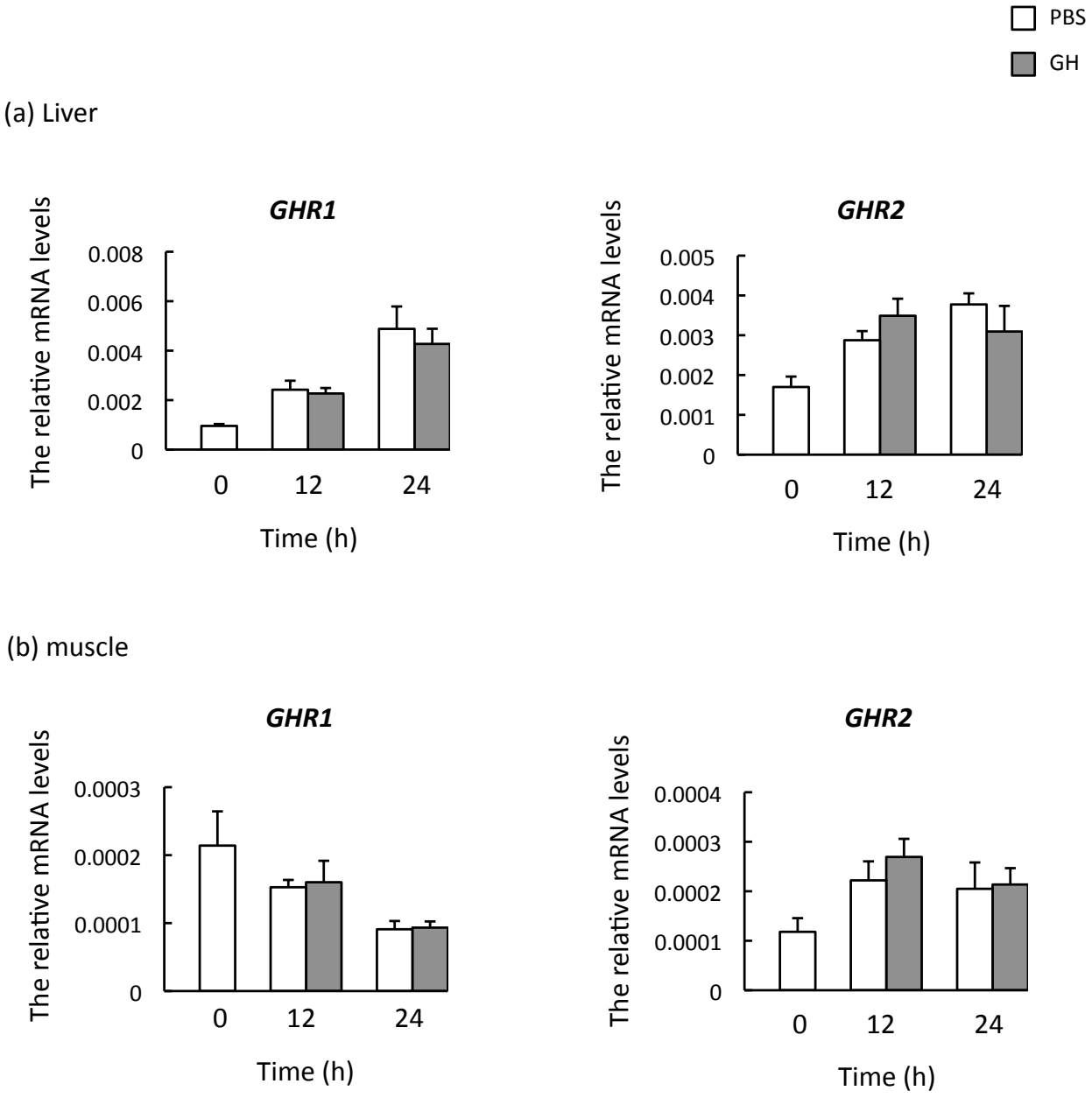


**Fig. 2-5.** The relative mRNA levels of torafugu GHR1 and GHR2 in torafugu (1) liver and (b) muscle slices treated with hyperosmolar PBS (white bars) and rtGH (5 ng/ml, dark bars).

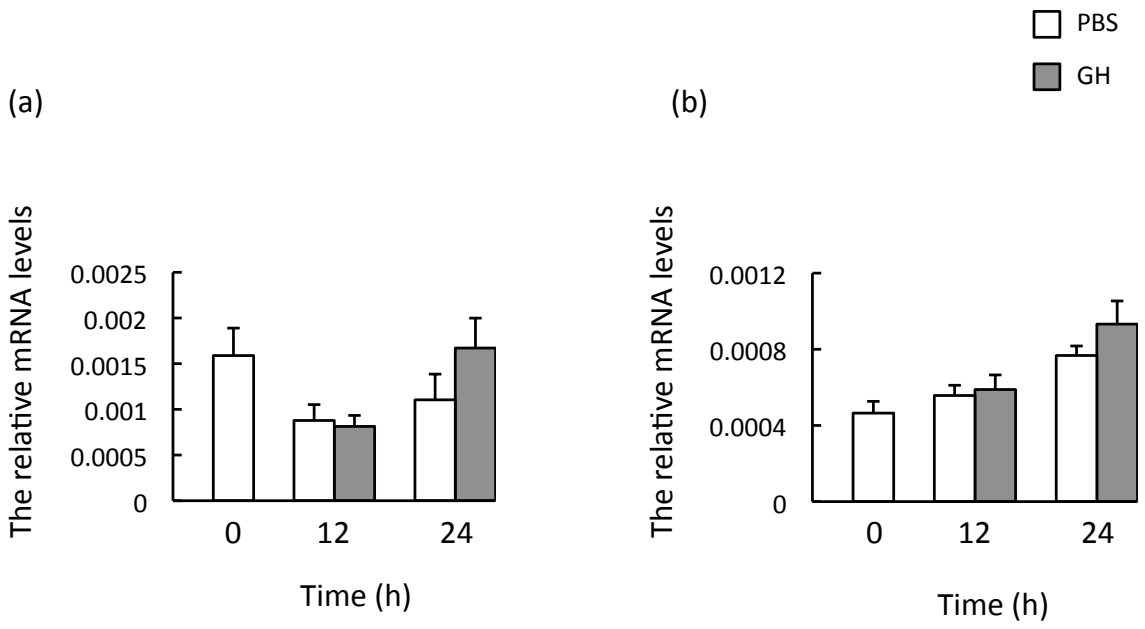


**Fig. 2-6.** The relative mRNA levels of torafugu IGF-I in torafugu liver slices treated with hyperosmolar PBS (white bars) and different concentrations of rtGH at (a) 5 ng/ml and (b) 200 ng/ml (dark bars).

## Chapter 3

## Comparison of TAG distribution among tissues and serum lipoproteins between torafugu and red seabream

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。5 年以内に出版予定。

## General discussion

Fish species are widely distributed over the world oceans. Their habitats are diverse and they have adapted to the environment by modifying their survival strategies including swimming performance (Yamanoue et al., 2010). The fish species having high swimming activity, such as red seabream and Atlantic salmon, always use their trunk body to move, whereas the fish species lying on the bottom of the ocean, such as torafugu and flounder, mainly use their fins to generate thrust. These behavioral differences lead to the differences of the energy metabolism. The species-specific distribution pattern of lipid accumulation is thought to be one of the phenotype reflecting these differences. With the recent progresses in fish research, the mechanisms of lipid metabolism have been demonstrated by molecular biological approaches. However, most of these studies were performed in fatty fish species. To increase the knowledge of the molecular mechanisms of lipid metabolism with comparison to lean fish species will help us to understand the more basic aspects of it.

GH is known to regulate the growth efficiency in vertebrates. This valuable hormone also has the role of maintaining energy metabolism in living body with its lipolytic effect in fatty fish as well as in mammals (Sheridan, 1994). In lean fish, however, there is less study on GH function of its lipid metabolism regulation. Therefore, in this study, the molecular mechanisms of GH on lipid metabolism in torafugu, a typical lean fish species, were investigated.

Torafugu belongs to the family Tetraodontidae, which includes more than 120 species of pufferfish. The extremely low lipid ratio of muscle to liver has been observed in several pufferfish species such as torafugu (Ando et al., 1993), four species of Indian marine puffer fish (Ghosh et al., 2005), and obscure puffer *Takifugu obscurus* (Liu et al., 2013). In Chapter 3, the TAG contents in liver and muscle in torafugu showed similar distribution patterns with total lipid contents (Kaneko et al., 2013). Our preliminary data on TAG contents in purple puffer *Takifugu porphyreus* and green spotted pufferfish

*Tetraodon nigroviridis* also revealed that both pufferfish accumulated less than 0.5% of TAG content in muscle and approximately 30% in liver as well as torafugu. These results suggest that the low lipid ratio of muscle to liver reflects TAG distribution among tissues as a common feature of pufferfish.

The present study indicated that torafugu modulate their lipid metabolism in part with the function of GH. In Chapter 1, rtGH showed the gene expression regulation at tissue-dependent manner, including lipid metabolism-related genes. Torafugu have two types of GHRs, GHR1 and GHR2, and the tissue distributions of their transcripts were unique to torafugu, namely, skeletal muscle had the highest gene expression level of GHR1. Actually, the preliminary data on tissue distributions of two types of GHRs in green spotted pufferfish also showed the similar expression patterns (data not shown), suggesting that this is conserved among pufferfish. Since rtGH repressed the gene expression of lipogenesis enzyme in muscle *ex vivo*, torafugu GH could strongly attenuate the lipogenesis in muscle under natural condition because of the high abundance of GHR1 *in vivo* as revealed in Chapter 2. Meanwhile, rtGH showed the gene regulations that possibly result in the NEFA reduction by inhibiting its uptake and *de novo* synthesis followed by oxidation in liver *ex vivo*. Besides, the higher concentration of rtGH stimulation induced the apolipoprotein gene expression, maybe involving the lipoprotein secretion. These results suggest that torafugu GH regulates the whole-body lipid balance in a concentration dependent manner (Fig. 4-1).

Although the rtGH treatments at the concentrations used in the present study significantly changed the several lipid metabolism-related gene expressions in liver *ex vivo*, the expression of IGF-I did not show any change by rtGH stimulation irrespective of concentration and incubation time. In many fatty fish species, the GH treatment induces the hepatic gene expression of IGF-I at the comparative concentration with the present study (Pierce et al., 2004; Leung et al., 2008; Pedroso et al., 2009; Pierce et al., 2011; Reindl et al., 2011). Taking these reports into account, the results obtained in this study suggest that torafugu liver has low capacity to produce IGF-I in response to GH stimulation. Histologically, the

liver consists of different cell types, parenchymal and non-parenchymal cells. The IGF-I gene expression is observed only in parenchymal cells in mammal (Uchijima et al., 1995). Patients of non-alcoholic fatty liver, whose parenchymal cells in liver accumulate lipids at high levels, show the low levels of circulating IGF-I (Arturi et al., 2011), suggesting the low capacity of producing IGF-I in fatty liver. These features are similar to those observed in torafugu liver. In fish, both the IGF-I production and lipid droplets accumulation are observed in liver (Eppler et al., 2007; Akiyoshi and Inoue, 2004), but the detailed localizations have not been identified. Since torafugu liver showed the high TAG content and mammalian adipose tissue-like response to rtGH stimulation (Oscarsson et al., 1996; Nam and Marcus, 2000; Louveau and Gondret, 2004; Hogan et al., 2005), there would be histological differences in torafugu liver compared to mammals. Further analysis for detailed identification of the localization of lipid droplets will assess this hypothesis.

In Chapter 3, the TAG content of torafugu serum was revealed to be comparative to that of red seabream. The TAG distribution among lipoproteins analyzed by HPLC showed the higher TAG content in torafugu TAG-rich lipoprotein fraction than that in red seabream. There may be two reasons causing this difference. One is the weak incorporation of TAG into torafugu muscle as discussed in Chapter 3. The TAG in TAG-rich lipoproteins is hydrolyzed by LPL function localized on the surface of endothelial cells of tissues. The previous study showed that the LPL mediates the selective uptake of TAG-rich lipoprotein through receptor-mediated endocytosis (Merkel et al., 1998). Although the VLDL receptor distribution in torafugu has not been confirmed yet, the extremely low TAG content in its muscle is possibly caused by the low level of LPL1 gene expression (Kaneko et al., 2013). To confirm this, the comparison of the enzymatic activity of LPL in muscle between torafugu and red seabream should be performed. The other reason for the high TAG content in TAG-rich lipoprotein fraction in torafugu might be an enhanced release of TAG-rich lipoprotein from torafugu liver. In the present study, torafugu rtGH stimulated the apolipoprotein gene expression in liver slices. The liver had low capacity to express the

IGF-I gene in response to GH. Previous studies showed that IGF-I has a role as a negative feedback regulator for GH secretion in fish pituitary as in mammals (Perez-Sanchez et al., 1992; Fruchtman et al., 2000). Therefore, in torafugu, the negative feedback by IGF-I could be attenuated and it results in the high concentration of circulating GH, enhancing the apolipoprotein expression in liver. This second hypothesis, however, leads a contradiction with the lipolytic effects of rtGH revealed in this study. Since torafugu accumulates high amounts of TAG in liver, the basal lipogenesis activity should be high. To get rid of this, some previous studies have to be cited. Liver-specific IGF-I deletion leads the elevated both basal levels of GH and insulin, an anabolic peptide hormone produced by pancreas (Sjögren et al., 2002). Insulin enhances lipogenesis activity and increases the TAG content of isolated hepatocytes in catfish *Pelteobagrus fulvidraco* (Zhuo et al., 2014). In this study, microarray analysis indicated that the rtGH stimulation does not change the expression level of insulin receptor in torafugu liver (data not shown). These information implies the possibility that the low IGF-I production in torafugu liver causes the elevation of both GH and insulin, the former regulates the lipoprotein secretion and the latter keeps the basal lipogenesis activity. Taken together, the TAG content in torafugu serum, which is comparative to that in red seabream, is possibly kept by the attenuated uptake into muscle and/or the enhanced lipoprotein secretion from liver (Fig. 4-2). To assess these hypotheses, it is essential to confirm the concentration of GH and insulin in torafugu circulation. The VLDL releasing capacity of torafugu and red seabream also should be compared *in vivo*.

The present study strongly indicates that torafugu has unique GH/GHR signaling compared to the fatty fish species. Whether this is a common feature in lean fish or not is unclear because of the less study on GH and GHR characteristics of lean fish species. Exceptionally, the studies of GH/GHR signaling in fine flounder *Paralichthys adspersus*, one of the typical lean fish, showed that the serum concentration of GH is not exceeding 10 ng/ml under normal feeding condition in several fish species belonging to fatty fish (Kajimura et al., 2004; Pierce et al., 2005; Gabillard et al., 2006; Shimizu et al.,

2009). On the other hand, fine flounder contains the higher basal level of circulating GH (100 to 170 ng/ml) together with low IGF-I level (Fuentes et al., 2012a and b). The tissue distribution of GHR in this species is determined only for GHR1 by semi-quantitative way (Fuentes et al., 2008). Therefore it is difficult to compare the tissue distribution pattern of GHR between fine flounder and torafugu. Interestingly, Fuentes et al. (2012a) revealed by Western blot analysis that fine flounder contains high amount of truncated GHR, a short form of GHR lacking the intracellular domain, in muscle. It is known in mammalian species that the GHR gene produces not only the full length GHR but also the short forms of GHR by posttranscriptional or posttranslational modifications, such as GH binding protein (GHBP) and truncated GHR (Dastot et al., 1998). The GHBP is a soluble form of GHR circulating in blood stream to regulate the half-life of GH (Veldhuis et al., 1993). The truncated GHR is a short membrane-anchored form of GHR lacking the intracellular domain that has the dominant-negative function for transmitting GH signaling (Ahmed et al., 2011). Fuentes et al. (2012a) confirmed that the abundance of truncated GHR is almost 4-fold higher than full length GHR, possibly resulting in the local GH resistance. GHBP and truncated GHR are identified in several fish species (Marchant et al., 1999; Perez-Sanchez et al., 2002; Fuentes et al., 2013; Einarsdottir et al., 2014) but not yet in torafugu. In this study, because the tissue distributions of GHR transcripts in torafugu were analyzed by using the gene-specific primers designed based on the extracellular domain sequences, the ratio of full length and truncated GHRs is unclear. Since this ratio will affect the actual GH signaling of each tissue, further investigations for the ratio of full length and truncated GHRs are necessary for understanding the GH/GHR signaling in torafugu.

In conclusion, this study revealed that the extremely low lipid ratio of muscle to liver in torafugu reflects the difference of TAG distribution, the neural lipids used as energy sources, although the TAG transport capacity by lipoproteins is higher than that of red seabream, which contains significantly higher TAG level in its muscle than torafugu. The cDNA cloning of two types of torafugu GHR and their

tissue distribution analyses confirmed that both torafugu GHR1 and GHR2 transcripts were ubiquitously expressed in various tissues. Especially, the highest mRNA level of GHR1 was observed in its muscle. The rtGH was demonstrated to exert the suppression effect of lipogenesis in muscle *ex vivo*. These results indicate that GH/GHR signaling is involved in the low TAG contents in torafugu muscle. Meanwhile, the rtGH also showed the lipolytic effects in liver *ex vivo*. A high concentration of rtGH enhanced the apolipoprotein expression, possibly related to the TAG-rich lipoprotein secretion. The whole body lipid balances in torafugu would be regulated in a GH-dependent manner.

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