MOLECULAR EVOLUTION OF GPCRS

Ghrelin/ghrelin receptors

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Abstract

After the discovery in 1996 of the GH secretagogue-receptor type-1a (GHS-R1a) as an orphan G-protein coupled receptor, many research groups attempted to identify the endogenous ligand. Finally, Kojima and colleagues successfully isolated the peptide ligand from rat stomach extracts, determined its structure, and named it ghrelin. The GHS-R1a is now accepted to be the ghrelin receptor. The existence of the ghrelin system has been demonstrated in many animal classes through biochemical and molecular biological strategies as well as through genome projects. Our work, focused on identifying the ghrelin receptor and its ligand ghrelin in laboratory animals, particularly nonmammalian vertebrates, has provided new insights into the molecular evolution of the ghrelin receptor. In mammals, it is assumed that the ghrelin receptor evolution is in line with the plate tectonics theory. In contrast, the evolution of the ghrelin receptor in nonmammalian vertebrates differs from that of mammals: multiplicity of the ghrelin receptor isoforms is observed in nonmammalian vertebrates only. This multiplicity is due to genome duplication and polyploidization events that particularly occurred in Teleostei. Furthermore, it is likely that the evolution of the ghrelin receptor is distinct from that of its ligand, ghrelin, because only one ghrelin isoform has been detected in all species examined so far. In this review, we summarize current knowledge related to the molecular evolution of the ghrelin receptor in mammalian and nonmammalian vertebrates.

Key Words
- ghrelin
- ghrelin receptor
- GHS-R
- GHS-R-like receptor

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The discovery of growth hormone secretagogue receptor and its endogenous ligand, ghrelin, and ghrelin gene-derived peptides

Most receptors for peptide hormones are G-protein-coupled receptors (GPCRs). There are still unidentified endogenous ligands for more than 140 GPCRs, and such receptors are termed as orphan GPCRs (Civelli 2012, Tang *et al*. 2012). Reverse pharmacology has been a successful approach to identify natural ligands, including ghrelin, for many orphan GPCRs (Kojima *et al*. 1999, Civelli *et al*. 2006). Ghrelin was identified as an endogenous ligand for the growth hormone secretagogue-receptor (GHS-R) type-1a (GHS-R1a), which was first discovered as an endogenous receptor for the artificial GH-releasing peptide (GHRP; Howard *et al*. 1996, Tannenbaum & Bowers 2001).

Actually, Howard *et al*. (1996) identified two GHS-R molecules with some variation in length, in both humans and pigs. In humans, one of these two is the functional receptor; this one is called the GHS-R1a and consists of 366 amino acids (AAs) with seven transmembrane domains (TMDs 1–7) (Howard *et al*. 1996). The other type is the alternative splice variant of the *GHS-R* gene named GHS-R1b, which consists of 289 AAs with TMDs 1–5 of GHS-R1a

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and a part of the connected intron. Only the GHS-R1a induces the intracellular Ca^{2+} signaling that mediates the activation of a G-protein subtype, $G\alpha_{q/11}$, by agonist treatment (Howard *et al*. 1996, Kojima *et al*. 1999, Wettschureck *et al*. 2005). The GHS-R1b does not induce Ca^{2+} signaling due to the lack of TMDs 6 and 7. Furthermore, the *GHS-R* belongs to a family with paralogue receptors such as the motilin, neuromedin U, and neurotensin, and still orphan GPCR, GPR39, according to their fundamental structural features (Fig. 1). All ligands for these receptors, including ghrelin, are known to be involved in gastrointestinal functions by binding to its own receptor, but note that the ligand for GPR39 is as yet unknown (Kojima *et al*. 2005).

Normally, GHS-R1a is considered to form a functional homodimer (Holst *et al*. 2005). However, a heterodimerization with GHS-R1b has been shown to occur and to reduce the signaling capacity of GHS-R1a (Chan & Cheng 2004, Leung *et al*. 2007, Chow *et al*. 2012), suggesting a dominant-negative role for GHS-R1b in GHS-R1a signaling (Leung *et al*. 2007). Furthermore, the GHS-R1a can pair with other GPCRs, e.g., melanocortin 3 receptor (MC3), dopamine receptors (D1 and D2), serotonin 2C receptor (5-HT2C), and somatostatin receptor-5 (SSTR5), or with members of the prostanoid receptor family such as prostacycline receptor, prostaglandin E_2 receptor subtype EP3-I, and the thromboxane A2 receptor (see review by Schellekens *et al*. (2013)). These heterodimerizations will affect ligand selectivity, G-protein coupling, and the downstream signaling of each receptor. Also the GHS-R1b can form heterodimers with other GPCRs, e.g., the neurotensin receptor 1 (Takahashi *et al*. 2006).

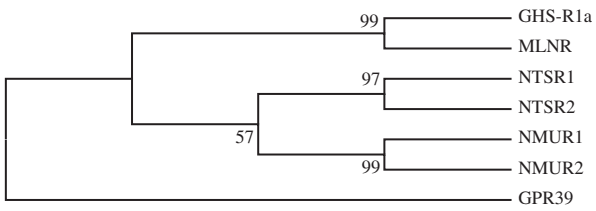

Figure 1
Molecular phylogenetic tree of the ghrelin receptor (GHS-R1a) family in humans. The phylogenetic tree of amino acid (AA) sequences was constructed by using the neighbor-joining (NJ) method with MEGA4 (http://www.megasoftware.net/). The numbers on the branch points are the bootstrap values (as percentages based on 1000 replicates). AA sequences obtained from the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Abbreviations and accession numbers indicate as follows: MLNR: motilin receptor (NM_001507), NMUR1 and 2: neuromedin-U receptor 1 and 2 (NM_006056 and NM_020167), and NTSR1 and 2: neurotensin receptor 1 and 2 (NM_002531 and NM_012344), and GPR39 (NM_001508).

Mammalian ghrelin generally consists of 28 AAs (Kojima *et al*. 1999), but the number of constituting AAs varies when nonmammalian vertebrates are included: 16 AAs in the elasmobranch stingray, 25 AAs in sharks, 17–23 AAs in teleosts, 27–28 AAs in amphibians, 25 AAs in a reptilian turtle, and 26 AAs in birds (Kaiya *et al*. 2011*a*,*b*). The N-terminal third serine residue of ghrelin is generally acylated with *n*-octanoic acid, and this acyl modification is essential for binding of ghrelin to the GHS-R1a and for eliciting the subsequent ghrelin activities (Kojima *et al*. 1999, Großauer *et al*. 2010). However, the acylated AA is substituted with threonine instead of serine in frogs of the genus *Rana*, and the threonine residue is acylated with *n*-octanoic or *n*-decanoic acid (Kaiya *et al*. 2001, 2011*a*,*b*). Various acyl modifications other than *n*-octanoylation, including various saturated and unsaturated medium-chain fatty acids, have been identified in both mammals and nonmammalian vertebrates (see review by Kojima *et al*. (2008)). In addition, phylogenetic analyses of ghrelin including both mammals and nonmammalian vertebrates reveal several structural features of ghrelin apart from the variation of the number of constituting AAs: i) high conservation of the N-terminal seven AA sequence, GSSFLSP, across species, ii) great diversity of AA sequence at the C-terminal side after the conserved sequence, iii) glycosylation in addition to acylation of ghrelin in the elasmobranch stingray, and iv) a C-terminal amidation unique for teleosts (see review by Kaiya *et al*. 2008, 2011*b*).

Mainly based on mammalian studies, it has been recognized that ghrelin is a multifunctional hormone involved in GH secretion, appetite regulation, neuroendocrine function, cardiovascular functioning, gastroenteropancreatic function, gastrointestinal motility, glucose metabolism, cell differentiation, immune function, bone metabolism, sleep, and the promotion of learning and memory (Diano *et al*. 2006, Hosoda *et al*. 2006, Chen *et al*. 2009, Carlini *et al*. 2010, Kojima & Kangawa 2010, Verhulst & Depoortere 2012). Our main aim is to explore what the general actions of ghrelin are in vertebrates. At present, it has been revealed that GH-releasing ability is a common action among those vertebrates examined so far, although effects on feeding regulation and gastrointestinal motility vary in each animal (see reviews by Kaiya *et al*. (2013*b*)). Further studies are required to clarify the fundamental roles of ghrelin in vertebrates.

Three bioactive peptides are generated from the ghrelin precursor: ghrelin, unacylated ghrelin (des-acyl ghrelin), and obestatin (Nishi *et al*. 2011). Ghrelin is produced by acylation of unacylated ghrelin with the

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specific enzyme ghrelin-*O*-acyltransferase (Gutierrez *et al.* 2008, Yang *et al.* 2008). Unacylated ghrelin is also present in circulating blood and the stomach, and the quantity is much greater than that of ghrelin (Kojima *et al.* 1999, Hosoda *et al.* 2000). Unacylated ghrelin exerts some biological actions, e.g., regulate feeding (Asakawa *et al.* 2005, Toshinai *et al.* 2006, Inhoff *et al.* 2009) and gut motility (Fujimiya *et al.* 2012), a GHS-R1a-independent antagonistic effect on ghrelin-induced insulin secretion and glucose metabolism, a trophic and protective effect on β-cells, as well as a role in muscle regeneration and in decreasing fat mass (Delhanty *et al.* 2012, Delhanty & van der Lely 2013). How does unacylated ghrelin act? It is clear that unacylated ghrelin does not induce an intracellular Ca^{2+} increase (Kojima *et al.* 1999). Granata *et al.* (2010) reported that unacylated ghrelin binds on pancreatic β-cells with high affinity even though GHS-R1a is not expressed, suggesting possible presence of a yet unidentified specific receptor for unacylated ghrelin.

Mammalian ghrelin receptors and speculation about their receptor evolution

As the AA sequences of numerous mammalian ghrelin receptors are available from genome sequencing projects, we have constructed a molecular phylogenetic tree based on these sequences (Fig. 2). The analysis shows a high sequence identity (85–95%) across different mammalian species. In addition, while investigating the regularity, we found that the classification of the mammals into clades on the basis of AA sequence of the ghrelin receptor is in agreement with classifications of the mammals based on plate tectonics theory and DNA sequence (Eizirik *et al.* 2001, Murphy *et al.* 2001*a*,*b*, Nishihara *et al.* 2009), although the accuracy of our analysis is still low. In this regard, we found three large groups, i.e., Euarchontoglires, Laurasiatheria, and Marsupialia (Fig. 2). Other than these, there are categories of Afrotheria and Xenarthra in the classifications of mammals based on plate tectonics theory. However, as the numbers of the species for Afrotheria and Xenarthra for which the ghrelin receptor sequences are known are still few, the accuracy of our analysis is low. For example, lesser hedgehog tenrec and African elephant actually belong to Afrotheria, but they were classified into different unexpected clades in this analysis (open squares, Fig. 2). In addition, because only partial sequences for Xenarthra were publicly available, we did not include these data in our analysis. Although more detailed studies are necessary in the future, current data imply that the evolution of the mammalian ghrelin receptor is the consequence of dispersal of animals with the continental drift.

Nonmammalian ghrelin receptors and their isoforms

In this section, we summarize what is known about the nonmammalian ghrelin receptor today. First, we give a brief description of different kinds and arrangements of nonmammalian ghrelin receptors before discussing their evolution. This is because the ghrelin receptors in nonmammals are more complicated/complex and diverse than in mammals.

Both the ghrelin receptor (GHS-R1a) and the alternative splice variant GHS-R1b are present in nonmammalian species. Unlike GHS-R1b, other alternative splice variants are found in birds, as will be described later. Most ghrelin receptors in nonmammalian species have been identified in fish (20 species). In other species, they have been found in three species of amphibians, two species of reptilians, and five species of aves. For further details, we would like to refer to our recent review (Kaiya *et al.* 2013*a*).

When comparing the primary structure of the ghrelin receptor protein in nonmammals, we find two interesting features. One is the presence of orthologous isoforms with different structural properties, i.e., GHS-Ra and GHS-R1a-like receptor (GHS-R1a-LR). The other is the isoforms that may have occurred through whole-genome duplication (WGD) or polyploidization limited to teleost fish.

GHS-Ra, meaning the GHS-R type-a, is the umbrella term for two ghrelin receptor isoforms: GHS-R1a and GHS-R2a. These two receptor isoforms are considered to be derived by a WGD event. A limited numbers of teleost fishes such as Cypriniformes (e.g., goldfish, carp, and zebrafish) and Siluriformes (e.g., channel catfish) have GHS-R1a and 2a. The AA sequences of GHS-R2a share ~70% identity with those of GHS-R1a (Small *et al.* 2009, Kaiya *et al.* 2010). The two isoforms are encoded by separate genes, e.g., the zebrafish *GHS-R1a* and *GHS-R2a* genes are located separately on chromosomes 4 and 24 respectively. This is in contrast to tetrapods including mammals, birds, reptiles, and amphibians, which have the GHS-R1a only.

Another orthologous isoform of the ghrelin receptor is GHS-R1a-LR. We designated this when discovered the receptor in Mozambique tilapia and rainbow trout (Kaiya *et al.* 2009*a*,*b*). GHS-R1a-LRs have unique features. One is that the second extracellular loop (ECL2) that connects TMDs 4 and 5 is notably longer when compared with that of the GHS-Ra (for a review, see Kaiya *et al.* (2013*a*)).

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Another is that an intracellular Ca^{2+} increase in response to ghrelin or GHSs is not confirmed in GHS-R1a-LR (Kaiya *et al*. 2009*a*,*b*), although pharmacological doses could increase intracellular Ca^{2+} of mammalian cells expressing GHS-R1a-LR in pufferfish and black porgy (Palyha *et al*. 2000, Chan & Cheng 2004). This dissimilarity between the GHS-Ra and GHS-R1a-LR is also evident in the phylogenetic relationship based on their AA sequences (Fig. 3). We can see that only a limited number of fish classified as Percomorpha within the superorder Acanthopterygii have GHS-R1a-LR, i.e., Perciformes such as black porgy (*Acanthopagrus schlegelii*) and tilapia (*Oreochromis mossambicus*); Gasterosteiformes such as stickleback (*Gasterosteus aculeatus*) and medaka (*Oryzias latipes*); Tetraodontiformes such as pufferfishes (*Spheroides nephelus*, *Tetraodon nigroviridis*, and *Takifugu rubripes*); and Salmoniformes such as rainbow trout (*Oncorhynchus mykiss*). The common denominators among these are that they are the most evolutionally advanced groups of teleost fishes.

Furthermore, there is an isoform that may have occurred by polyploidization in the GHS-Ra and GHS-R1a-LR. This has been found in a few species of teleost fish and shows much higher identity (95%) when compared with the identity (70%) of isoforms occurred by genome duplication such as GHS-Ra. A representative species that has the isoform is goldfish. Goldfish has two ghrelin receptor isoforms that occurred by genome duplication: GHS-R1a and GHS-R2a. In addition to these, each receptor has the isoforms that may be occur through polyploidization, namely GHS-R1a-1 and 1a-2, and GHS-R2a-1 and 2a-2. Each receptor originates from a separate gene (Kaiya *et al*. 2010). This type of isoform is also found in GHS-R1a-LR of the rainbow trout i.e., the DQTA/LN-type and ERAT/IS-type GHS-R1a-LR (Kaiya *et al*. 2009*b*). The names of these isoforms indicate AA substitutions at D20E Q32R T54A A62T L168I and N264S (denoted as AA followed by AA position). It has been demonstrated that these two AA sequences are derived from at least three

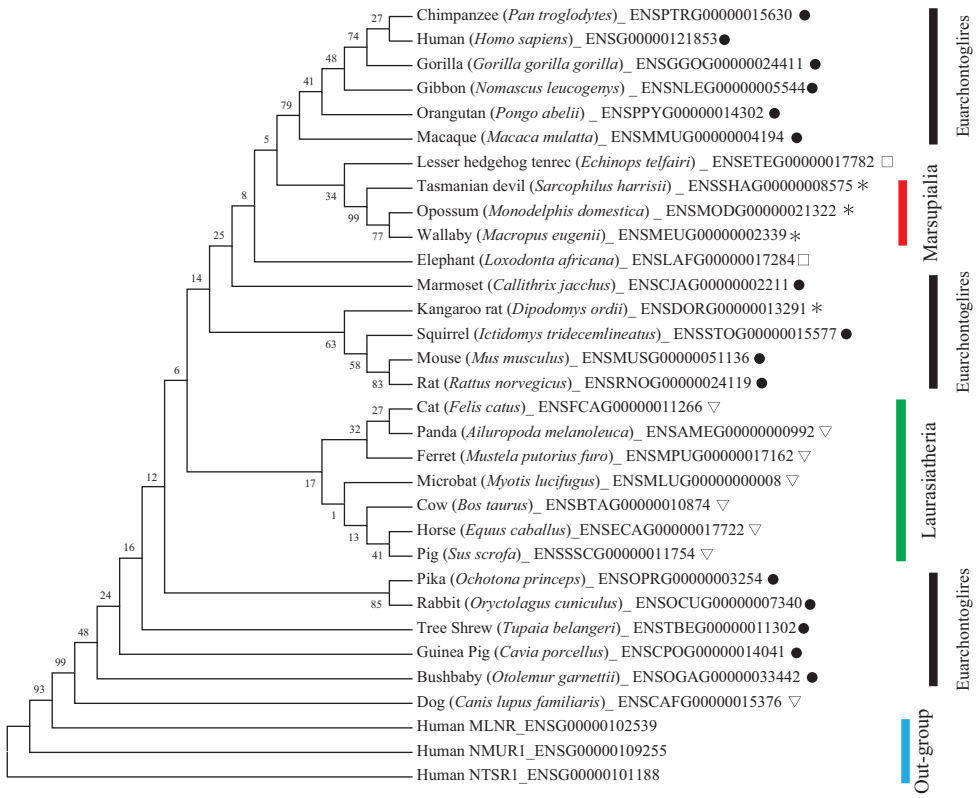

Figure 2

Molecular phylogenetic tree of the ghrelin receptor (GHS-R1a) in mammals. The phylogenetic tree of amino acid (AA) sequences was constructed by using the neighbor-joining (NJ) method with MEGA4 Software (http://www.megasoftware.net/). The numbers on the branch points are the bootstrap values (as percentages based on 1000 replicates). AA sequences obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html). GeneID shows the following species name. Receptors for human motilin (MLNR), neuromedin-U (NMUR1), and neurotensin (NTSR1) were used as the out group. Symbols are defined as follows: closed circle, Euarchontoglires; open square, Afrotheria; asterisk, Marsupialia; open down triangle, Laurasiatheria.

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distinct genes: the ERAT/IS-type originates from one gene and the DQTA/LN-type derives from two separate genes (Kaiya *et al*. 2009*b*). Thus the constitutions of the ghrelin receptors in nonmammalian vertebrates are more complicated than their mammalian counterparts.

Multiplicity of nonmammalian ghrelin receptors

The multiplicity of nonmammalian ghrelin receptors in a limited number of fish is the result of genome polyploidization, in which there are two patterns. One is the substitution of ~30% of the AA sequence, as seen in the GHS-R1a and GHS-R2a isoforms in goldfish, zebrafish, and channel catfish (Olsson *et al*. 2008, Small *et al*. 2009, Kaiya *et al*. 2010). We currently believe that this pattern originates from a chromosomal duplication after 3R-WGD (Meyer & Schartl 1999, Jaillon *et al*. 2004). The other pattern is the substitution of only 5% of the AA sequence, as seen between goldfish GHS-R1a-1 and 1a-2, between goldfish GHS-R2a-1 and 2a-2, and between rainbow trout GHS-R1a-LRs. We speculate that this pattern is derived by genetic recombination after a frame-shift mutation that occurred in the paternal or maternal allele. As an exception, however, polyploidization of the ghrelin receptor does not seem to have occurred in Perciformes such as tilapia, although tilapia experienced 3R-WGD (Kaiya *et al*. 2009*b*). Current data are too scarce to provide a conclusive explanation for the lack of polypoidization in this species.

Distribution of the ghrelin receptors and the difference between mammals and nonmammalian vertebrates

In mammals, the distribution of the ghrelin receptor is most extensively studied in laboratory mammals. Although a widespread distribution of the ghrelin receptor has been demonstrated, the highest levels of expression (ghrelin receptor mRNA) has been detected in the pituitary gland (Gnanapavan *et al*. 2002, Ueberberg *et al*. 2009), which is consistent with the role of ghrelin in the regulation of GH release. In general, the ghrelin receptor transcripts have also been detected in brain areas linked to energy homeostasis such as the hypothalamus, hippocampus, substantia nigra, ventral tegmental area, and dorsal and median raphe nuclei (Bennett *et al*. 1997, Guan *et al*. 1997, Kageyama *et al*. 2005, Zigman *et al*. 2006, Chen *et al*. 2009), although clear species differences in the distribution, e.g., between lemurs and rats, have been reported (Mitchell *et al*. 2001). The ghrelin receptor mRNA is also detected in various peripheral tissues such as the thyroid, heart, lung, liver, kidney, pancreas, stomach, spleen, intestine, adrenal gland, testis, and adipose tissue (Gnanapavan *et al*. 2002, Barreiro *et al*. 2003, Dass *et al*. 2003, Kageyama *et al*. 2005, Camiña 2006, Sun *et al*. 2007, Kitazawa *et al*. 2011).

The GHS-R1b splice variant of the ghrelin gene shows a different pattern of expression when compared with that of the GHS-R1a. The highest expression was found in the skin, followed by the myocardium, pituitary, thyroid gland, and pancreas of humans (Gnanapavan *et al*. 2002). It has been suggested that GHS-R1b plays a role for the trafficking of the GHS-R1a to the cell surface (Leung *et al*. 2007), but this widespread and different/graded levels of expression among tissues suggest an unknown physiological relevance of GHS-R1b in each tissue.

What about the tissue distribution of the ghrelin receptor in nonmammalian vertebrates? Similar to mammals, the GHS-R1a or GHS-R1a-LR transcripts have been found in various brain regions and peripheral organs, and the pituitary is the predominant expressing site for the ghrelin receptor isoforms in the majority of species, e.g. the channel catfish (Small *et al*. 2009), chickens (Geelissen *et al*. 2003, Tanaka *et al*. 2003, Saito *et al*. 2005, Richards *et al*. 2006, Yamamoto *et al*. 2008), and ducks (Nie *et al*. 2009) for GHS-R1a, and in the black porgy (Chan & Cheng 2004), orange-spotted grouper (Chen *et al*. 2008), and rainbow trout (Kaiya *et al*. 2009*b*) for GHS-R1a-LR. An exception is frogs, where *GHS-R1a* mRNA is not detected in the pituitary but mainly in the brain (Kaiya *et al*. 2011*a*). Thus, ghrelin receptor expression in the pituitary gland is not a dominant feature in all nonmammalian species.

The brain is the tissue showing the second highest expression of the ghrelin receptor in fish and birds. In addition, the ghrelin receptor gene expression has also been detected in various amounts in more or less all peripheral tissues, such as the eyes, heart, thymus, liver, stomach, intestine, spleen, gill, gall bladder, muscle, kidney, head kidney, Brockmann bodies, skin, muscle, and gonads for fish (Chan & Cheng 2004, Chen *et al*. 2008, Kaiya *et al*. 2009*a*,*b*, Small *et al*. 2009, Cruz *et al*. 2010), the stomach and gonads, and to a lesser extent in the small and large intestines, adrenal gland, and kidney in frogs (Kaiya *et al*. 2011*a*), and the heart, lung, thymus, liver, spleen, pancreas, gastrointestinal tract, adrenal gland, kidney, gonads, breast muscle, subcutaneous fat, leg muscle, abdominal fat, and uropygial gland in birds (Geelissen *et al*. 2003, Tanaka *et al*. 2003, Saito *et al*. 2005, Richards *et al*. 2006, Kitazawa *et al*. 2009, Nie *et al*. 2009). In birds, strain differences (Geelissen *et al*. 2003, Tanaka

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et al. 2003, Richards & McMurtry 2010) and a region-specific expression in the gastrointestinal tract (Kitazawa *et al*. 2009) have been reported. In summary, these data indicate that ghrelin acts on various organs in non-mammalian vertebrates as it does in mammals.

Cypriniformes fish such as goldfish and zebrafish, as well as Siluriformes such as channel catfish, have paralogous *GHS-Ra*, *GHS-R1a* and *2a*, showing different expression levels and tissue patterns (Small *et al*. 2009, Cruz *et al*. 2010, Kaiya *et al*. 2010). This suggests different mechanisms underlying the regulation of the expression of these genes.

The presence of *GHS-R1b* or an expected receptor, *GHS-R1b-LR*, in nonmammalian vertebrates has been reported: the *GHS-R1b-LR* mRNA has been detected in various brain regions of the black porgy whereas only a low expression was measured in peripheral tissues (Chan & Cheng 2004). In rainbow trout and channel catfish, *GHS-R1b* mRNA is strongly expressed in the pituitary, whereas a weak expression is observed in other peripheral organs (Kaiya *et al*. 2009*b*, Small *et al*. 2009). This is different from the results reported for mammals (Gnanapavan *et al*. 2002). On the other hand, in Mozambique tilapia, ghrelin receptor transcripts are detected in the stomach, adipose tissue, gill, liver, intestine, spleen, kidney, and muscle as well as in the brain (Kaiya *et al*. 2009*a*). Likewise, in orange-spotted grouper, the *GHS-R1b* mRNA is detected in various peripheral organs as well as in the brain and pituitary (Chen *et al*. 2008).

In birds, there is a splice variant which is different from GHS-R1b in structure, namely the GHS-R1aV. The gene of this variant is expressed in almost all tissues of chickens, and the expression pattern is almost identical to that of GHS-R1a (Geelissen *et al*. 2003, Tanaka *et al*. 2003, Richards & McMurtry 2010). Other splice variants, the GHS-Rtv and GHS-Rtv-like receptor, show a limited and specific expression in the ovary of chickens (Sirotkin *et al*. 2006) and in the proventriculus and gizzard of the Japanese quail (Kitazawa *et al*. 2009) respectively. For further information about the structure of these receptor variants in birds, we would like to refer to a detailed review (Kaiya *et al*. 2013*a*).

Evolution of ghrelin receptors in vertebrates with focus on nonmammalian vertebrates

We prepared a phylogenetic tree for the ghrelin receptors identified so far, and we will use it in this study to speculate about the evolution of the ghrelin receptor in vertebrates including both mammals and nonmammals (Figs. 3 and 4).

In a search of the Ensemble database (http://www.ensembl.org/Petromyzon_marinus/Info/Index/), a partial AA sequence with 50% identity to human GHS-R1a was detected in sea lamprey (*Petromyzon marinus*), which belongs to the group Cyclostomata in the class Agnatha, a class of fish with the characteristics of ancient basal vertebrates. This receptor could not be placed in any branch of GHS-Ra or GHS-R1a-LR when the phylogenetic analysis was carried out (Fig. 3). Therefore, the receptor of the sea lamprey may have the ancestral characteristics of the ghrelin receptor.

Gnathostomes are divided into Chondrichthyes and Osteichthyes (Fig. 4). In Chondrichthyes, genome-decoding efforts have focused on the elephant shark (*Callorhinchus milii*; http://esharkgenome.imcb.a-star.edu.sg). In a search of the database, we found a partial receptor sequence similar to human GHS-R1a with 51% identity. The characteristics of the elephant shark receptor are similar to those of GHS-Ra rather than the GHS-R1a-LR. Osteichthyes have split into Actinopterygii (the ray-finned fish lineage) and Sarcopterygii (the lobe-finned fish lineage) during evolution (Fig. 4). Sarcopterygii includes coelacanths, the family of fish that led to tetrapods. In a search of the Ensembl database for coelacanths (http://www.ensembl.org/Latimeria_chalumnae/Info/Index/), a receptor sequence that has similar characteristics to GHS-R1a was found. This is in line with the presence of the GHS-R1a in all tetrapods. On the other hand, in Actinopterygii, two types of the ghrelin receptor, GHS-Ra and GHS-R1a-LR are present. Actinopterygii where the ghrelin receptor has been identified comprised four classes of fish: Perciformes, Salmoniformes, Cypriniformes, and Siluriformes (Fig. 4). Perciformes and Salmoniformes have the GHS-R1a-LR, and Cypriniformes and Siluriformes have the GHS-Ra. Furthermore, Salmoniformes and Cypriniformes have paralogous isoforms that occurred through polyploidization. Why do Actinopterygian fish have two types of the ghrelin receptor? What is the difference from other group? One answer may lie in a common characteristic that we noticed in species with the GHS-Ra: they have swim bladders that have evolved from lungs as outlined below.

Primitive Teleostei had lungs, and these evolved into swim bladders in some Teleostei (Farmer 1997, Zaccone *et al*. 2012). In Polypteriformes, Semionotiformes, and Amiiformes, which are primitive Actinopterigii, and lungfish, which belong to Sarcopterygii, swim bladders perform pulmonary respiration separately from gill breathing and so they function as the lungs. In contrast, Acipenseriformes and Teleostei have complete swim

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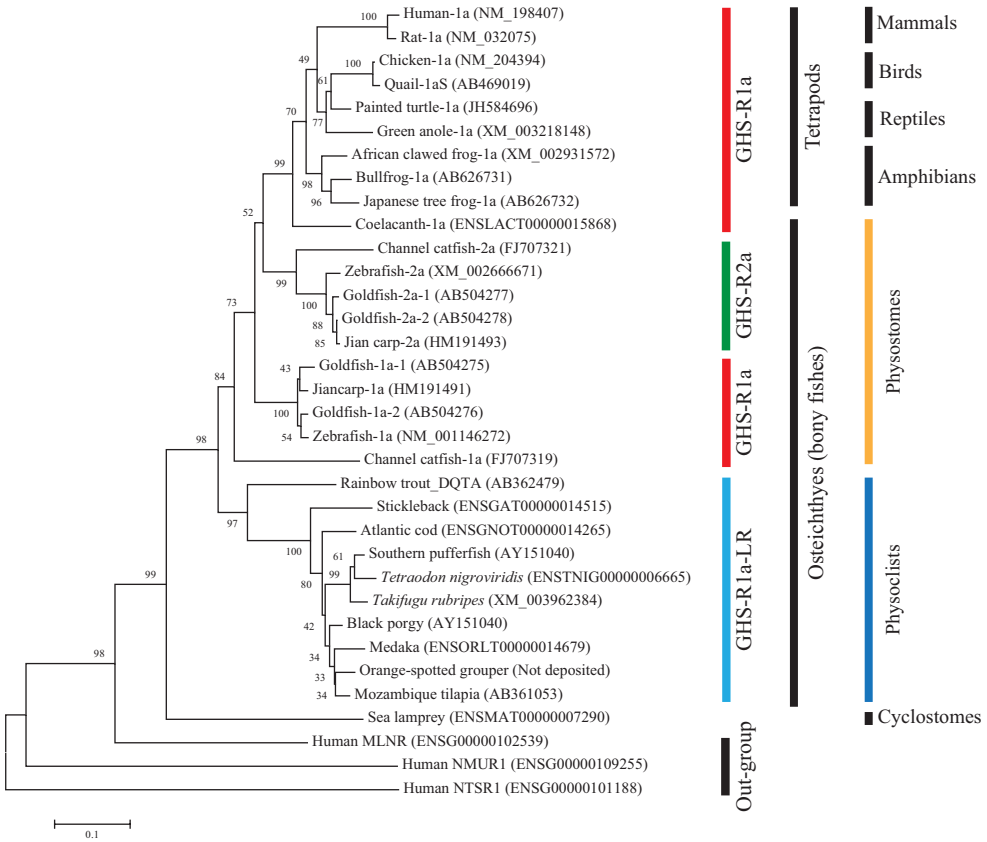

Figure 3

Molecular phylogenetic tree of GHS-Ra and GHS-R1a-LR in nonmammalian vertebrates. The phylogenetic tree of amino acid (AA) sequences was constructed by using the neighbor-joining (NJ) method with MEGA4 (http://www.megasoftware.net/). The numbers on the branch points are the bootstrap values (as percentages based on 1000 replicates). The scale bar indicates the average number of substitutions per position (a relative measure of evolutionary distance). AA sequences obtained from the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the Ensembl Genome Browser (http://www.ensembl.org/index.html). Accession number of the gene or GeneID shows the following species name. Receptors for human motilin (MLNR), neuromedin-U (NMUR1), and neurotensin (NTSR1) were used as the out group.

bladders, which have lost the breathing function. Species with the GHS-R1a-LR share a morphological characteristic: i.e., the lack of a connection between the swim bladder and the alimentary canal. Such fish are called 'physoclistous' fish, and Perciformes and Salmoniformes are included. In contrast, Cypriniformes and Siluriformes, which have the GHS-Ra, have a pneumatic duct connecting the swim bladder to the alimentary canal. These fish are called 'physostomous' fish. Therefore, teleosts that have swim bladders derived from lungs and tetrapods, which have lungs, have the GHS-Ra isoform. For some reason, in 'physoclistous' fish that have developed complete swim bladders during the evolutional process, structures of the GHS-Ra have changed leading to the ghrelin receptor GHS-R1a-LR. We speculate that a reason that different forms of the ghrelin receptor are present in the ray-finned fish lineage (Actinopterygii), but not the lobe-finned fish (Sarcopterygii), might be an involvement of the third round of 3R-WGD that occurred only in Actinopterygii lineage (Meyer & Schartl 1999, Jaillon *et al*. 2004). A complication is that highly similar receptor isoforms are present in goldfish (e.g., GHS-R1a-1 and 1a-2) or rainbow trout (DQTA-type and ERAT-type) (Kaiya *et al*. 2009*b*, 2010). It may be hypothesized that their polyploidization event that occurred after 3R-WGD (Leggatt & Iwama 2003), and a tandem duplication of the genes, as occurred in the opsin gene in these species (Rennison *et al*. 2012) may be responsible for these new traits. Among Euteleosts, it is speculated that the presence of multiple paralogoue isoforms may be a peculiar characteristic for Ostariophysi and Protacanthopterygii (Meyer & Schartl 1999, Jaillon *et al*. 2004), and further studies are necessary to clarify this issue on the ghrelin receptor.

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Comparison of the ghrelin receptor signaling

Current knowledge shows that the intracellular signaling triggered by the ghrelin receptor follows the general pattern that the ghrelin receptor activates a G-protein subtype, $G\alpha_{q/11}$, which induces the production of inositol triphosphate (IP3), which releases Ca^{2+} from intracellular calcium stores, whereas diacylglycerol activates protein kinase C (PKC) (Howard *et al.* 1996, Wettschureck *et al.* 2005). These events are not only seen in cells transfected with GHS-R1a but also in pituitary somatotrophs (Cheng *et al.* 1991, Herrington & Hille 1994, Lei *et al.* 1995, Bresson-Bépoldin & Dufy-Barbe 1996, Lania *et al.* 1998). In neuropeptide Y-containing neurons, ghrelin-induced Ca^{2+} increase is responsible for the calcium influx through N-type calcium channels via the cAMP- protein kinase A (PKA) signaling pathway through a G-protein coupled to the ghrelin receptor (Kohno *et al.* 2003). In porcine somatotropes, the three distinct second messenger systems, such as adenylyl cyclase/PKA, phospholipase C (PLC)/PKC, and extracellular Ca^{2+} systems, are sequentially involved in the ghrelin response (Malagón *et al.* 2003).

On the other hand, according to the review paper of Camiña (2006), ghrelin activates MAPK through mediating the Ras-Raf-MEK-MAPK pathway through activation of a tyrosine kinase receptor in adrenal cells. In addition, another mechanism through which ghrelin may activate MAPK is via PI3 kinase and PLC through $G_{i/o}$ as shown in 3T3-L1 cells. Furthermore, in hepatoma cells, ghrelin has been shown to increase MAPK activity via the association of growth factor receptor-bound protein 2 with insulin receptor substrate-1 and PI3 kinase. These three MAPK pathways are associated with stimulation of cell proliferation. On the other hand, ghrelin exerts an inhibitory effect on angiogenic factors such as fibroblast growth factor-2 (Conconi *et al.* 2004).

Interestingly, the GHS-R1a shows a constitutive activity (high-basal IP3 production) in the absence of agonists (Herrington & Hille 1994, Lania *et al.* 1998, Holst *et al.* 2005). This activity causes a PLC–PKC-dependent Ca^{2+} mobilization that is associated with the L-type voltage-gated calcium channel. The basal PLC as well as the extracellular signal-regulated kinase 1 and 2 activity is activated or inhibited by GHRP-6 and a GHS-R antagonist, [D-Lys3]-GHRP-6 respectively (Chu *et al.* 2007).

Nonmammalian ghrelin receptors have been successfully expressed in mammalian cells, and these show a rise in intracellular Ca^{2+} upon stimulation with ghrelin or GHSs (Palyha *et al.* 2000, Chan & Cheng 2004, Chan *et al.* 2004, Kitazawa *et al.* 2009, Kaiya *et al.* 2010, 2011*a*, Tachibana *et al.* 2011). A similar Ca^{2+} mobilization was also observed in goldfish somatotrophs and gonadotrophs in the pituitary (Grey & Chang 2009, 2013, Grey *et al.* 2010), which are responsible for the release of GH and luteinizing hormone (LH) respectively. The signaling pathways have been gradually clarified: the ghrelin-induced GH and LH release from goldfish pituitary cells are regulated by nitric oxide signaling (Grey & Chang 2013), and PKA and PKC differently regulate it (Grey & Chang 2011). On the other hand, in fish-specific *GHS-R1a-LRs* expressing cells found in the pufferfish and black porgy (Palyha *et al.* 2000, Chan & Cheng 2004), Ca^{2+} signaling is activated by GHSs, but relatively high doses of receptor agonists are required when the dose was compared with that required to stimulate the GHS-Ra. In addition, no Ca^{2+} signaling could be detected in GHS-R1a-LRs expressing cells of tilapia and rainbow trout, even though when homologous ghrelin was used (Kaiya *et al.* 2009*a*,*b*). As described earlier, these receptors have a specific structural feature such as the long ECL2 (Kaiya *et al.* 2013*a*). Further studies are needed to elucidate the relationship between ghrelin signaling mechanisms and receptor structures involved in the expression of the activity.

Ghrelin receptor evolution, ligand selectivity, and receptor functionality to ghrelin

The evolution of a new endocrine function occurs with the acquisition of a new physiological function and the establishment of a new ligand–receptor system controlling this function. In general, because a hormone does not have any bioactivity itself without binding to its receptor, if the hormone evolves but a receptor does not evolve to bind the hormone, no new functionality will arise. This theory predicts that coevolution of the ligand and its receptor needs to occur to produce new functionality and raises the question whether coevolution has occurred in the case of ghrelin and the ghrelin receptor.

During the long history of ghrelin receptor evolution, the part of the receptor that is least likely to change is the structure that participates in the ligand binding. Both nonmammalian and mammalian ghrelin receptors are capable of binding synthetic GHSs such as GHRP2, GHRP6, ipamorelin, L163,255, L692,585, L163,540, and hexarelin (for a review, see Kaiya *et al.* (2008, 2011*b*)). Interestingly, the degree of agonistic activity of each artificial GHS varies according to the receptor isoform present in the animal, indicating that the structural interactions between the ligand and receptor that are essential for receptor activation did not change during the evolution of vertebrates.

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Feighner *et al.* (1998) reported on the relationship between certain AA residues and agonist binding to human GHS-R1a. The AA residues D99, C116, E124, M213, S217, and H280, have crucial roles in receptor activation. In particular, M213, S217, and H280 are required for the binding of GHRP6 and L692,585. The above six AA residues are conserved in GHS-Ra or GHS-R1a-LR identified in all nonmammalian vertebrates except stickleback (Kaiya *et al.* 2013*a*). This suggests that both the GHS-Ra and GHS-R1a-LR in nonmammalian vertebrates have the ability to bind GHRP6. However, GHS-R1a-1, GHS-R1a-2, and GHS-R2a-2 in goldfish selectively bind GHRP6 or hexarelin (Kaiya *et al.* 2010, 2013*a*), and GHS-R1a-LRs from rainbow trout and tilapia do not show Ca^{2+} response to GHRP6 in transfected HEK293 or CHO cells at all (Kaiya *et al.* 2009*a*,*b*). Thus, the interaction between the agonist and key AA residues in the receptor related to agonist binding may be more complicated than anticipated by Feighner *et al.* (1998). The minimum essential structure of ghrelin for binding to the receptor is the first four AAs (GSSF) including the acyl modification (Bednarek *et al.* 2000, Matsumoto *et al.* 2001).

As mentioned earlier, ghrelin receptor shows strong, ligand-independent constitutive signaling in transfected COS7 or HEK293 cells in addition to ligand-dependent Ca^{2+} signaling (Holst *et al.* 2003). In the case of human GHS-R1a, it has been reported that V160, F279, A204, I134, and A204 are important AA residues for the constitutive receptor activity, i.e., distinct sets of AAs are involved in ligand binding and constitutive activity (Holst

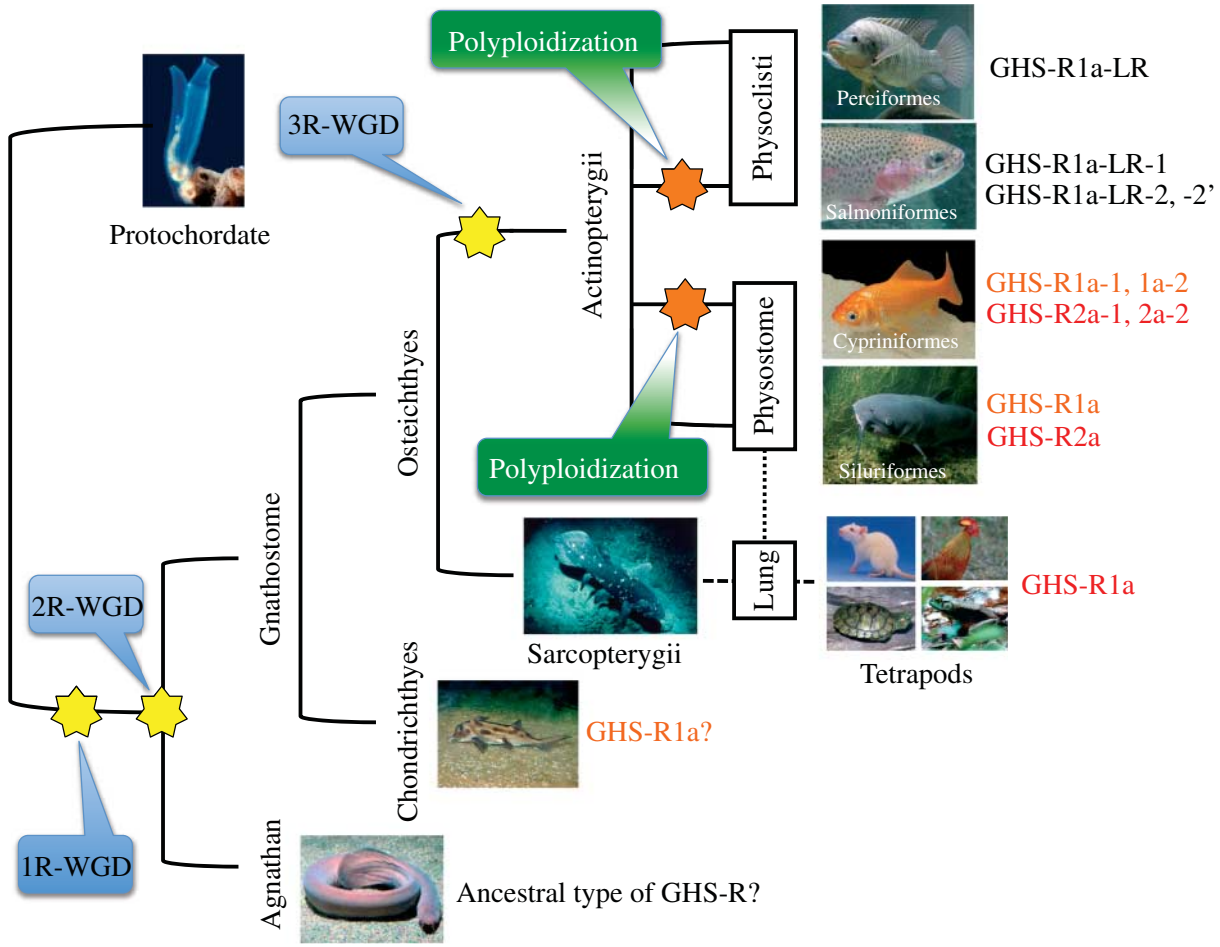

Figure 4

Schematic diagram of the evolution of the ghrelin receptor in vertebrates. The scheme includes data for Cyclostomata (Agnathans) and elephant shark (Chondrichthyes). The question marks indicate the possibility that an amino acid fragment of their receptor is GHS-R1a or an ancestral type of GHS-R. The three whole-genome duplication (WGD) events are shown by yellow stars. Polyploidization that occurred in some teleost species of Actinopterygii is shown by orange stars. The GHS-Ra and its isoforms are found in physostomous fish, which have the anlage of lungs, and tetrapods, which have lungs. In contrast, the GHS-R1a-LR and its isoforms are found in physoclistious fish, which have swim bladders that are not connected to the alimentary tract.

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et al. 2004, Liu *et al*. 2007, Rediger *et al*. 2011). Because these AA residues do not substitute and are conserved in the GHS-Ra and GHS-R1a-LR isoforms identified in nonmammalian vertebrates (Kaiya *et al*. 2013*a*), it is presumed that constitutive activity has been conserved in the evolution of the ghrelin receptor, although the only nonmammalian species where this has been confirmed is in the receptor for the black porgy (Leung *et al*. 2007).

Did ghrelin receptor and ghrelin peptide coevolve?

In mammals, one form of ghrelin peptide and its receptor are present. However, as studies on nonmammalian vertebrates show that multiple forms of ghrelin and the ghrelin receptors exist, they have revealed that the evolution of the ghrelin receptor is more complicated than we first thought.

As described earlier, a plurality of the ghrelin receptors has been found, although only in a limited number of teleost lineages such as Salmoniformes, Cypriniformes, and Siluriformes, in which polyploidization of the genome has occurred. The multiplicity results in two patterns of genome polyploidization, as described earlier: chromosomal duplication by 3R-WGD and the genetic recombination after a frame-shift mutation that may occur in the paternal or maternal allele.

Now, the question then arises whether ghrelin, the ligand for the receptor, also duplicated or if an increased number of isoforms is associated with multiplicity of the receptor. Consequently, there is no evidence for multiple ghrelin genes, which means a multiple number of the ghrelin sequence, in any species (Fig. 5; Kaiya *et al*. 2011*b*). This suggests that the evolution of the ghrelin receptor and its ligand, ghrelin, occurred independently, and that evolutionary pressures were applied to the receptor gene only. Consequently it may be speculated that a coevolution

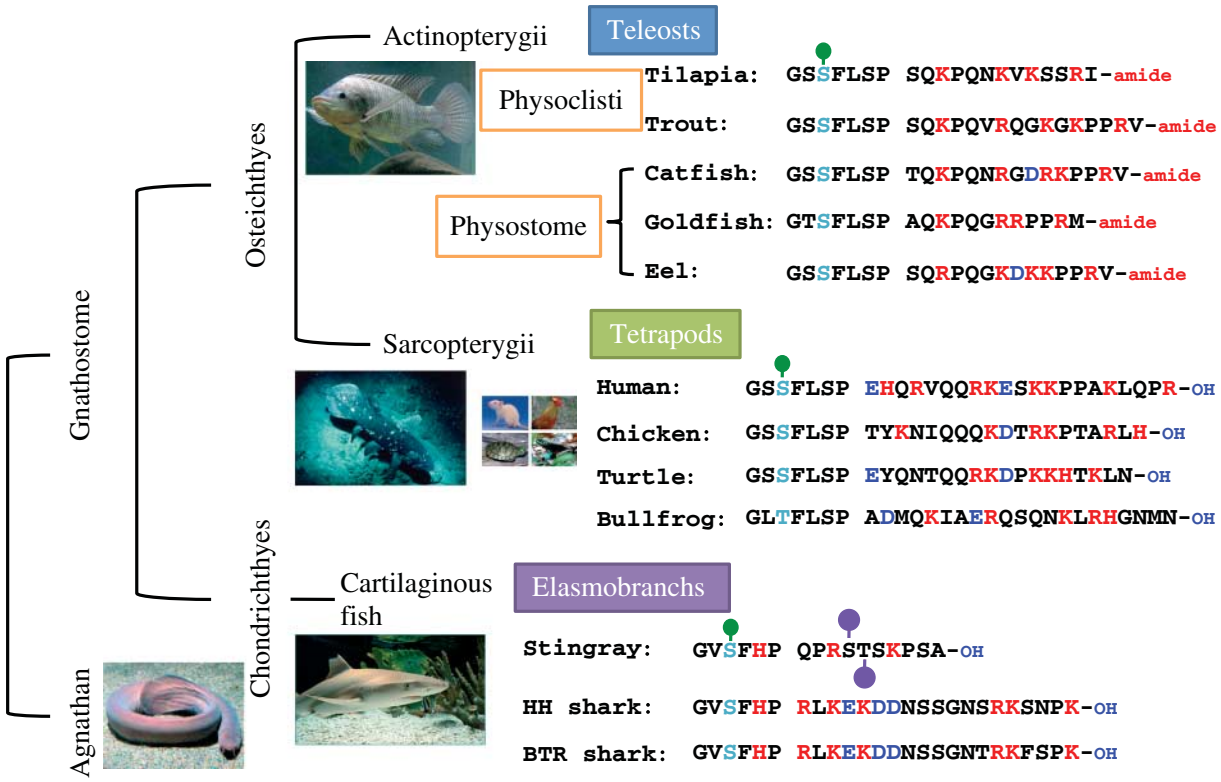

Figure 5

Schematic diagram of the evolution of ghrelin. In elasmobranchs, HH shark, and BTR shark denote hammerhead shark and blacktip reef shark respectively. In each ghrelin amino acid (AA) sequence, light blue letters indicate AAs that have fatty acid modifications, and red or blue letters indicate AAs that have a positive or negative charge respectively. The carboxyl terminus of ghrelin in teleosts is amidated. Fatty acid modification is also indicated with a green symbol on the first sequence in each group. In elasmobranchs, in addition to the acyl modification, ghrelin-like peptide of stingray has an additional modification by mucin-type carbohydrate chains, as shown in the purple symbols (Kaiya *et al*. 2009*c*). Some ghrelin molecules have truncated C-termini due to the post-translational processing. However, the presence of isoforms with AA substitutions has not been identified, even in fish species where polyploidization of the ghrelin receptor is seen.

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of the ghrelin receptor and ghrelin has not occurred. Interestingly, some evidence suggests that multiplicity (evolution) of the ghrelin receptor may accompany the change in ligand selectivity, as outlined below.

In ghrelin receptors for goldfish (Kaiya *et al.* 2010), GHS-R2a does not show any ligand selectivity between goldfish ghrelin and GHSs such as GHRP-6 and hexarelin. In contrast, GHS-R1a shows ligand selectivity for activation of the receptor; GHRP-6 does not elicit an increase in intracellular Ca^{2+} through GHS-R1a. This finding suggests that the numerous differences in constructing AA residues between GHS-R1a and GHS-R2a affect GHRP-6 selectivity, even though the key AAs for GHRP-6 binding to GHS-R1a have not been changed during receptor evolution, as mentioned earlier (Feighner *et al.* 1998). The reason why goldfish GHS-R1a shows ligand selectivity can be speculated on as follows. In the phylogenetic tree analysis (Fig. 3), GHS-R2a, but not GHS-R1a, for Cyprinoformes and Siliuriformes were classified in the same clade as tetrapod GHS-R1a. We believe that this may be because of the historical naming of these receptors. The zebrafish GHS-R1a was the first GHS-Ra isoform identified in fish species (Olsson *et al.* 2008). Thereafter, our group discovered the existence of a receptor isoform for GHS-R1a in zebrafish in a search of the NCBI database and designated it GHS-R2a (for a review, see Kaiya *et al.* (2008)). Therefore, primarily GHS-R2a of Teleostei has the same feature as tetrapod GHS-R1a, which does not show ligand selectivity, and rather retains the basic features of the ghrelin receptor. In contrast, the current GHS-R1a of Teleostei may be an 'evolved' type of the ghrelin receptor and show ligand selectivity. Furthermore, evolutionally advanced fishes do not have GHS-Ra but have GHS-R1a-LR, although receptor activation and ligand-binding capacity have not been confirmed in some fish species after ghrelin treatment (Kaiya *et al.* 2009*a*,*b*). Thus, it can be speculated that the ghrelin receptor may be in the middle of evolving independently from ghrelin (Fig. 4).

Conclusion

In this review, we have discussed the evolution of the ghrelin receptor in vertebrates and shown that differences in the structure and function of the ghrelin receptor in various vertebrate species may relate to the formation of lungs, a physoclistous swim bladder, or a physostomous swim bladder. The physostome, which has a pneumatic duct connecting the swim bladder to the alimentary canal, is a vestigial character derived from ancestral fish that had lungs. Thus, the presence of GHS-Ra in physostomous fish and tetrapods, but not physoclistous fish, suggests that GHS-Ra is a rather old type of the ghrelin receptor. In contrast, another type of GHS-Ra, the GHS-R1a-LRs, for which we could not confirm a functional activity using a mammalian cell expression system, is present only in a limited number of teleosts. These are the evolutionally advanced species in terms of both morphology and function, being physoclistous fish. Thus, GHS-R1a-LR should be a more recently developed type of ghrelin receptor. However, the relationship between the function and evolution of lungs, and ghrelin's function is unknown. Further studies are necessary to clarify the physiological relevance of the multiplicity of the ghrelin receptor in some teleost species, and the possibility of the presence of another ligand, which means a ghrelin isoform or unknown peptide, for the multiple ghrelin receptors. Such knowledge would give us a deeper understanding of the significance of the ghrelin–ghrelin receptor system in vertebrates.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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