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

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, $\text{G}\alpha_{q/11}$, 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.* 2011a,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, 2011a,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 non-mammalian 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, 2011b).

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.* (2013b)). 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

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.* 2001a,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.* 2013a).

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.* 2009a,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.* (2013a)).

Another is that an intracellular Ca^{2+} increase in response to ghrelin or GHSs is not confirmed in GHS-R1a-LR (Kaiya *et al.* 2009a,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 (*Spheroideus 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.* 2009b). 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.

distinct genes: the ERAT/IS-type originates from one gene and the DQTA/LN-type derives from two separate genes (Kaiya *et al.* 2009b). 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.* 2009b). Current data are too scarce to provide a conclusive explanation for the lack of polyploidization 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.* 2009b) 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.* 2011a). 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.* 2009a,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.* 2011a), 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

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.* 2009b, 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.* 2009a). 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.* 2013a).

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 Ensembl 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 (*Callorhynchus 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 coelacanth, the family of fish that led to tetrapods. In a search of the Ensembl database for coelacanth (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, Zaccane *et al.* 2012). In Polypteriformes, Semionotiformes, and Amiiformes, which are primitive Actinopterygii, 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

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 evolutionary 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.* 2009b, 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 paralogous 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.

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-Bépolin & 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 somatotrophs, 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, 2011a,

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.* 2009a,b). As described earlier, these receptors have a specific structural feature such as the long ECL2 (Kaiya *et al.* 2013a). 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, 2011b)). 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.

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.* 2013a). 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, 2013a), 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.* 2009a,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 physoclistous fish, which have swim bladders that are not connected to the alimentary tract.

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.* 2013a), 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.* 2011b). 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.* 2009c). 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.

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.* 2009a,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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References

- Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM & Kasuga M 2005 Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* **54** 18–24. (doi:10.1136/gut.2004.038737)
- Barreiro ML, Suominen JS, Gaytán F, Pinilla L, Chopin LK, Casanueva FF, Diéguez C, Aguilar E, Toppari J & Tena-Sempere M 2003 Developmental, stage-specific, and hormonally regulated expression of growth hormone secretagogue receptor messenger RNA in rat testis. *Biology of Reproduction* **68** 1631–1640. (doi:10.1095/biolreprod.102.008862)
- Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH & Heck JV 2000 Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *Journal of Medicinal Chemistry* **43** 4370–4376. (doi:10.1021/jm0001727)
- Bennett PA, Thomas GB, Howard AD, Feighner SD, van der Ploeg LH, Smith RG & Robinson IC 1997 Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth

- hormone in the rat. *Endocrinology* **138** 4552–4557. (doi:10.1210/en.138.11.4552)
- Bresson-Bépalain L & Dufy-Barbe L 1996 GHRP6-stimulated hormone secretion in somatotrophs: involvement of intracellular and extracellular calcium sources. *Journal of Neuroendocrinology* **8** 309–314. (doi:10.1046/j.1365-2826.1996.04608.x)
- Camiña JP 2006 Cell biology of the ghrelin receptor. *Journal of Neuroendocrinology* **18** 65–76. (doi:10.1111/j.1365-2826.2005.01379.x)
- Carlini VP, Perez MF, Salde E, Schiöth HB, Ramirez OA & de Barioglio SR 2010 Ghrelin induced memory facilitation implicates nitric oxide synthase activation and decrease in the threshold to promote LTP in hippocampal dentate gyrus. *Physiology & Behavior* **101** 117–123. (doi:10.1016/j.physbeh.2010.04.026)
- Chan CB & Cheng CH 2004 Identification and functional characterization of two alternatively spliced growth hormone secretagogue receptor transcripts from the pituitary of black seabream, *Acanthopagrus schlegelii*. *Molecular and Cellular Endocrinology* **214** 81–95. (doi:10.1016/j.mce.2003.11.020)
- Chan CB, Leung PK, Wise H & Cheng CH 2004 Signal transduction mechanism of the seabream growth hormone secretagogue receptor. *FEBS Letters* **577** 147–153. (doi:10.1016/j.febslet.2004.08.088)
- Chen T, Tang Z, Yan A, Li W & Lin H 2008 Molecular cloning and mRNA expression analysis of two GH secretagogue receptor transcripts in orange-spotted grouper (*Epinephelus coioides*). *Journal of Endocrinology* **199** 253–265. (doi:10.1677/JOE-08-0325)
- Chen CY, Asakawa A, Fujimiya M, Lee SD & Inui A 2009 Ghrelin gene products and the regulation of food intake and gut motility. *Pharmacological Reviews* **61** 430–481. (doi:10.1124/pr.109.001958)
- Cheng K, Chan WW, Butler B, Barreto A Jr & Smith RG 1991 Evidence for a role of protein kinase-C in His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂-induced growth hormone release from rat primary pituitary cells. *Endocrinology* **129** 3337–3342. (doi:10.1210/endo-129-6-3337)
- Chow KB, Sun J, Chu KM, Tai Cheung W, Cheng CH & Wise H 2012 The truncated ghrelin receptor polypeptide (GHS-R1b) is localized in the endoplasmic reticulum where it forms heterodimers with ghrelin receptors (GHS-R1a) to attenuate their cell surface expression. *Molecular and Cellular Endocrinology* **348** 247–254. (doi:10.1016/j.mce.2011.08.034)
- Chu KM, Chow KB, Leung PK, Lau PN, Chan CB, Cheng CH & Wise H 2007 Over-expression of the truncated ghrelin receptor polypeptide attenuates the constitutive activation of phosphatidylinositol-specific phospholipase C by ghrelin receptors but has no effect on ghrelin-stimulated extracellular signal-regulated kinase 1/2 activity. *International Journal of Biochemistry & Cell Biology* **39** 752–764. (doi:10.1016/j.biocel.2006.11.007)
- Civelli O 2012 Orphan GPCRs and neuromodulation. *Neuron* **76** 12–21. (doi:10.1016/j.neuron.2012.09.009)
- Civelli O, Saito Y, Wang Z, Nothacker HP & Reinscheid RK 2006 Orphan GPCRs and their ligands. *Pharmacology & Therapeutics* **110** 525–532. (doi:10.1016/j.pharmthera.2005.10.001)
- Conconi MT, Nico B, Guidolin D, Baiguera S, Spinazzi R, Rebuffat P, Malendowicz LK, Vacca A, Carraro G, Parnigotto PP *et al.* 2004 Ghrelin inhibits FGF-2-mediated angiogenesis *in vitro* and *in vivo*. *Peptides* **25** 2179–2185. (doi:10.1016/j.peptides.2004.08.011)
- Cruz SA, Tseng YC, Kaiya H & Hwang PP 2010 Ghrelin affects carbohydrate-glycogen metabolism via insulin inhibition and glucagon stimulation in the zebrafish (*Danio rerio*) brain. *Comparative Biochemistry and Physiology – Part A, Molecular & Integrative Physiology* **156** 190–200. (doi:10.1016/j.cbpa.2010.01.019)
- Delhanty PJ & van der Lely AJ 2013 Ghrelin: a new treatment for non-alcoholic fatty liver disease? *Endocrine* **43** 247–248. (doi:10.1007/s12020-012-9800-2)
- Delhanty PJ, Neggers SJ & van der Lely AJ 2012 Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. *European Journal of Endocrinology* **167** 601–608. (doi:10.1530/EJE-12-0456)
- Dass NB, Munonyara M, Bassil AK, Hervieu GJ, Osbourne S, Corcoran S, Morgan M & Sanger GJ 2003 Growth hormone secretagogue receptors in rat and human gastrointestinal tract and the effects of ghrelin. *Neuroscience* **120** 443–453. (doi:10.1016/S0306-4522(03)00327-0)
- Diano S, Farr SA, Benoit SC, McNay EC, da Silva I, Horvath B, Gaskin FS, Nonaka N, Jaeger LB, Banks WA *et al.* 2006 Ghrelin controls hippocampal spine synapse density and memory performance. *Nature Neuroscience* **9** 381–388. (doi:10.1038/nn1656)
- Eizirik E, Murphy WJ & O'Brien SJ 2001 Molecular dating and biogeography of the early placental mammal radiation. *Journal of Heredity* **92** 212–219. (doi:10.1093/jhered/92.2.212)
- Farmer C 1997 Did lungs and the intracardiac shunt evolve to oxygenate the heart in vertebrates? *Paleobiology* **23** 358–372. (doi:10.1666/0094-8373-23.3.358)
- Feighner SD, Howard AD, Prendergast K, Palyha OC, Hreniuk DL, Nargund R, Underwood D, Tata JR, Dean DC, Tan CP *et al.* 1998 Structural requirements for the activation of the human growth hormone secretagogue receptor by peptide and nonpeptide secretagogues. *Molecular Endocrinology* **12** 137–145. (doi:10.1210/me.12.1.137)
- Fujimiya M, Ataka K, Asakawa A, Chen CY, Kato I & Inui A 2012 Regulation of gastroduodenal motility: acyl ghrelin, des-acyl ghrelin and obestatin and hypothalamic peptides. *Digestion* **85** 90–94. (doi:10.1159/000334654)
- Geelissen SM, Beck IM, Darras VM, Kühn ER & Van der Geyten S 2003 Distribution and regulation of chicken growth hormone secretagogue receptor isoforms. *General and Comparative Endocrinology* **134** 167–174. (doi:10.1016/S0016-6480(03)00250-8)
- Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB & Korbonits M 2002 The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *Journal of Clinical Endocrinology and Metabolism* **87** 2988–2991. (doi:10.1210/jc.87.6.2988)
- Granata R, Baragli A, Settanni F, Scarlatti F & Ghigo E 2010 Unraveling the role of the ghrelin gene peptides in the endocrine pancreas. *Journal of Molecular Endocrinology* **45** 107–118. (doi:10.1677/JME-10-0019)
- Grey CL & Chang JP 2009 Ghrelin-induced growth hormone release from goldfish pituitary cells involves voltage-sensitive calcium channels. *General and Comparative Endocrinology* **160** 148–157. (doi:10.1016/j.ygcen.2008.11.006)
- Grey CL & Chang JP 2011 Differential involvement of protein kinase C and protein kinase A in ghrelin-induced growth hormone and gonadotrophin release from goldfish (*Carassius auratus*) pituitary cells. *Journal of Neuroendocrinology* **23** 1273–1287. (doi:10.1111/j.1365-2826.2011.02221.x)
- Grey CL & Chang JP 2013 Nitric oxide signaling in ghrelin-induced LH release from goldfish pituitary cells. *General and Comparative Endocrinology* **183** 7–13. (doi:10.1016/j.ygcen.2012.11.022)
- Grey CL, Grayfer L, Belosevic M & Chang JP 2010 Ghrelin stimulation of gonadotropins (LH) release from goldfish pituitary cells: presence of the growth hormone secretagogue receptor (GHS-R1a) and involvement of voltage-sensitive Ca²⁺ channels. *Molecular and Cellular Endocrinology* **317** 64–77. (doi:10.1016/j.mce.2009.12.024)
- Großauer J, Kosol S, Schrank E & Zanger K 2010 The peptide hormone ghrelin binds to membrane-mimetics via its octanoyl chain and an adjacent phenylalanine. *Bioorganic & Medicinal Chemistry* **18** 5483–5488. (doi:10.1016/j.bmc.2010.06.062)
- Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghi DJ, Smith RG, Van der Ploeg LH & Howard AD 1997 Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Research and Molecular Brain Research* **48** 23–29. (doi:10.1016/S0169-328X(97)00071-5)
- Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE & Hale JE 2008 Ghrelin octanoylation mediated by an orphan lipid transferase. *PNAS* **105** 6320–6325. (doi:10.1073/pnas.0800708105)

- Herrington J & Hille B 1994 Growth hormone-releasing hexapeptide elevates intracellular calcium in rat somatotropes by two mechanisms. *Endocrinology* **135** 1100–1108. (doi:10.1210/endo.135.3.8070352)
- Holst B, Cygankiewicz A, Jensen TH, Ankersen M & Schwartz TW 2003 High constitutive signaling of the ghrelin receptor – identification of a potent inverse agonist. *Molecular Endocrinology* **17** 2201–2210. (doi:10.1210/me.2003-0069)
- Holst B, Holliday ND, Bach A, Elling CE, Cox HM & Schwartz TW 2004 Common structural basis for constitutive activity of the ghrelin receptor family. *Journal of Biological Chemistry* **279** 53806–53817. (doi:10.1074/jbc.M407676200)
- Holst B, Brandt E, Bach A, Hedding A & Schwartz TW 2005 Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling. *Molecular Endocrinology* **19** 2400–2411. (doi:10.1210/me.2005-0059)
- Hosoda H, Kojima M, Matsuo H & Kangawa K 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochemical and Biophysical Research Communications* **279** 909–913. (doi:10.1006/bbrc.2000.4039)
- Hosoda H, Kojima M & Kangawa K 2006 Biological, physiological, and pharmacological aspects of ghrelin. *Journal of Pharmacological Sciences* **100** 398–410. (doi:10.1254/jphs.CRJ06002X)
- Howard AD, Feighner SD, Cully DF, Arena JP, Liberatore PA, Rosenblum CL, Hamelin M, Hreniuk DL, Palyha OC, Anderson J *et al.* 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273** 974–977. (doi:10.1126/science.273.5277.974)
- Inhoff T, Wiedenmann B, Klapp BF, Mönnikes H & Kobelt P 2009 Is desacyl ghrelin a modulator of food intake? *Peptides* **30** 991–994. (doi:10.1016/j.peptides.2009.01.019)
- Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A *et al.* 2004 Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* **431** 946–957. (doi:10.1038/nature03025)
- Kageyama H, Funahashi H, Hirayama M, Takenoya F, Kita T, Kato S, Sakurai J, Lee EY, Inoue S, Date Y *et al.* 2005 Morphological analysis of ghrelin and its receptor distribution in the rat pancreas. *Regulatory Peptides* **126** 67–71. (doi:10.1016/j.regpep.2004.08.031)
- Kaiya H, Kojima M, Hosoda H, Koda A, Yamamoto K, Kitajima Y, Matsumoto M, Minamitake Y, Kikuyama S & Kangawa K 2001 Bullfrog ghrelin is modified by *n*-octanoic acid at its third threonine residue. *Journal of Biological Chemistry* **276** 40441–40448. (doi:10.1074/jbc.M105212200)
- Kaiya H, Miyazato M, Kangawa K, Peter RE & Unniappan S 2008 Ghrelin: a multifunctional hormone in non-mammalian vertebrates. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **149** 109–128. (doi:10.1016/j.cbpa.2007.12.004)
- Kaiya H, Mori T, Miyazato M & Kangawa K 2009a Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **153** 438–450. (doi:10.1016/j.cbpa.2009.04.612)
- Kaiya H, Riley LG, Janzen W, Hirano T, Grau EG, Miyazato M & Kangawa K 2009b Identification and genomic sequence of a ghrelin receptor (GHS-R)-like receptor in the Mozambique tilapia, *Oreochromis mossambicus*. *Zoological Science* **26** 330–337. (doi:10.2108/zsj.26.330)
- Kaiya H, Kodama S, Ishiguro K, Matsuda K, Uchiyama M, Miyazato M & Kangawa K 2009c Ghrelin-like peptide with fatty acid modification and O-glycosylation in the red stingray, *Dasyatis akajei*. *BMC Biochemistry* **10** 30. (doi:10.1186/1471-2091-10-30)
- Kaiya H, Miura T, Matsuda K, Miyazato M & Kangawa K 2010 Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2a in goldfish, *Carassius auratus*. *Molecular and Cellular Endocrinology* **327** 25–39. (doi:10.1016/j.mce.2010.06.004)
- Kaiya H, Koizumi Y, Konno N, Yamamoto K, Uchiyama M, Kangawa K & Miyazato M 2011a Ghrelin receptor in two species of Anuran Amphibian, Bullfrog (*Rana catesbeiana*), and Japanese tree frog (*Hyla japonica*). *Frontiers in Endocrinology* **2** 31. (doi:10.3389/fendo.2011.00031)
- Kaiya H, Miyazato M & Kangawa K 2011b Recent advances in the phylogenetic study of ghrelin. *Peptides* **32** 2155–2174. (doi:10.1016/j.peptides.2011.04.027)
- Kaiya H, Kangawa K & Miyazato M 2013a Ghrelin receptors in non-mammalian vertebrates. *Frontiers in Endocrinology* **4** 81. (doi:10.3389/fendo.2013.00081)
- Kaiya H, Kangawa K & Miyazato M 2013b What is the general action of ghrelin for vertebrates? – comparisons of ghrelin's effects across vertebrates *General and Comparative Endocrinology* **181** 187–191. (doi:10.1016/j.ygcen.2012.10.015)
- Kitazawa T, Maeda Y & Kaiya H 2009 Molecular cloning of growth hormone secretagogue-receptor and effect of quail ghrelin on gastrointestinal motility in Japanese quail. *Regulatory Peptides* **158** 132–142. (doi:10.1016/j.regpep.2009.07.005)
- Kitazawa T, Nakamura T, Saeki A, Teraoka H, Hiraga T & Kaiya H 2011 Molecular identification of ghrelin receptor (GHS-R1a) and its functional role in the gastrointestinal tract of the guinea-pig. *Peptides* **32** 1876–1886. (doi:10.1016/j.peptides.2011.07.026)
- Kohn D, Gao HZ, Muroya S, Kikuyama S & Yada T 2003 Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca²⁺ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* **52** 948–956. (doi:10.2337/diabetes.52.4.948)
- Kojima M & Kangawa K 2005 Ghrelin: structure and function. *Physiological Reviews* **85** 495–522. (doi:10.1152/physrev.00012.2004)
- Kojima M & Kangawa K 2010 Ghrelin: more than endogenous growth hormone secretagogue. *Annals of the New York Academy of Sciences* **1200** 140–148. (doi:10.1111/j.1749-6632.2010.05516.x)
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide. *Nature* **402** 656–660. (doi:10.1038/45230)
- Kojima M, Ida T & Sato T 2008 Structure of mammalian and nonmammalian ghrelins. *Vitamins and Hormones* **77** 31–46. (doi:10.1016/S0083-6729(06)77003-0)
- Lania A, Ballare E, Corbetta S, Filopanti M, Persani L & Spada A 1998 Growth hormone-releasing hexapeptide (GHRP-6) increases intracellular calcium concentrations in cultured cells from human pituitary adenomas of different types. *European Journal of Endocrinology* **139** 343–348. (doi:10.1530/eje.0.1390343)
- Leggatt RA & Iwama GK 2003 Occurrence of polyploidy in the fishes. *Reviews in Fish Biology and Fisheries* **13** 237–246. (doi:10.1023/B:RFBF.0000033049.00668.fe)
- Leung PK, Chow KB, Lau PN, Chu KM, Chan CB, Cheng CH & Wise H 2007 The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor. *Cellular Signalling* **19** 1011–1022. (doi:10.1016/j.cellsig.2006.11.011)
- Lei T, Buchfelder M, Fahlbusch R & Adams EF 1995 Growth hormone releasing peptide (GHRP-6) stimulates phosphatidylinositol (PI) turnover in human pituitary somatotroph cells. *Journal of Molecular Endocrinology* **14** 135–138. (doi:10.1677/jme.0.0140135)
- Liu G, Fortin JP, Beinborn M & Kopin AS 2007 Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities. *Journal of Pharmacology and Experimental Therapeutics* **322** 1036–1043. (doi:10.1124/jpet.107.123141)
- Malagón MM, Luque RM, Ruiz-Guerrero E, Rodríguez-Pacheco F, García-Navarro S, Casanueva FF, Gracia-Navarro F & Castaño JP 2003 Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. *Endocrinology* **144** 5372–5380. (doi:10.1210/en.2003-0723)
- Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y & Kangawa K 2001 Structure–activity relationship of ghrelin: pharmacological study of

- ghrelin peptides. *Biochemical and Biophysical Research Communications* **287** 142–146. (doi:10.1006/bbrc.2001.5553)
- Meyer A & Scharl M 1999 Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Current Opinion in Cell Biology* **11** 699–704. (doi:10.1016/S0955-0674(99)00039-3)
- Mitchell V, Bouret S, Beauvillain JC, Schilling A, Perret M, Kordon C & Epelbaum J 2001 Comparative distribution of mRNA encoding the growth hormone secretagogue-receptor (GHS-R) in *Microcebus murinus* (Primate, lemurian) and rat forebrain and pituitary. *Journal of Comparative Neurology* **429** 469–489. (doi:10.1002/1096-9861(20010115)429:3<469::AID-CNE8>3.0.CO;2-)
- Murphy WJ, Eizirik E, O'Brien SJ, Madsen O, Scally M, Douady CJ, Teeling E, Ryder OA, Stanhope MJ, de Jong WW *et al.* 2001a Resolution of the early placental mammal radiation using Bayesian phylogenetics. *Science* **294** 2348–2351. (doi:10.1126/science.1067179)
- Murphy WJ, Eizirik E, Johnson WE, Zhang YP, Ryder OA & O'Brien SJ 2001b Molecular phylogenetics and the origins of placental mammals. *Nature* **409** 614–618. (doi:10.1038/35054550)
- Nie Q, Fang M, Xie L, Peng X, Xu H, Luo C, Zhang D & Zhang X 2009 Molecular characterization of the ghrelin and ghrelin receptor genes and effects on fat deposition in chicken and duck. *Journal of Biomedicine & Biotechnology* **2009** 567120. (doi:10.1155/2009/567120)
- Nishi Y, Yoh J, Hiejima H & Kojima M 2011 Structures and molecular forms of the ghrelin-family peptides. *Peptides* **32** 2175–2182. (doi:10.1016/j.peptides.2011.07.024)
- Nishihara H, Maruyama S & Okada N 2009 Retroposon analysis and recent geological data suggest near-simultaneous divergence of the three superorders of mammals. *PNAS* **106** 5235–5240. (doi:10.1073/pnas.0809297106)
- Olsson C, Holbrook JD, Bompadre G, Jönsson E, Hoyle CH, Sanger GJ, Holmgren S & Andrews PL 2008 Identification of genes for the ghrelin and motilin receptors and a novel related gene in fish, and stimulation of intestinal motility in zebrafish (*Danio rerio*) by ghrelin and motilin. *General and Comparative Endocrinology* **155** 217–226. (doi:10.1016/j.ygcen.2007.05.016)
- Palyha OC, Feighner SD, Tan CP, McKee KK, Hreniuk DL, Gao YD, Schleim KD, Yang L, Morriello GJ, Nargund R *et al.* 2000 Ligand activation domain of human orphan growth hormone (GH) secretagogue receptor (GHS-R) conserved from Pufferfish to humans. *Molecular Endocrinology* **14** 160–169. (doi:10.1210/me.14.1.160)
- Rediger A, Piechowski CL, Yi CX, Tarnow P, Strotmann R, Grütters A, Krude H, Schöneberg T, Tschöp MH, Kleinau G *et al.* 2011 Mutually opposite signal modulation by hypothalamic heterodimerization of ghrelin and melanocortin-3 receptors. *Journal of Biological Chemistry* **286** 39623–39631. (doi:10.1074/jbc.M111.287607)
- Rennison DJ, Owens GL & Taylor JS 2012 Opsin gene duplication and divergence in ray-finned fish. *Molecular Phylogenetics and Evolution* **62** 986–1008. (doi:10.1016/j.ympev.2011.11.030)
- Richards MP & McMurtry JP 2010 The avian proghrelin system. *International Journal of Peptides* **2010** 749401. (doi:10.1155/2010/749401)
- Richards MP, Poch SM & McMurtry JP 2006 Characterization of turkey and chicken ghrelin genes, and regulation of ghrelin and ghrelin receptor mRNA levels in broiler chickens. *General and Comparative Endocrinology* **145** 298–310. (doi:10.1016/j.ygcen.2005.09.013)
- Saito E-S, Kaiya H, Tachibana T, Tomonaga S, Denbow DM, Kangawa K & Furuse M 2005 Inhibitory effect of ghrelin on food intake is mediated by the corticotropin-releasing factor system in neonatal chicks. *Regulatory Peptides* **125** 201–208. (doi:10.1016/j.regpep.2004.09.003)
- Schellekens H, Dinan TG & Cryan JF 2013 Taking two to tango: a role for ghrelin receptor heterodimerization in stress and reward. *Frontiers in Neuroscience* **7** 148.
- Sirotkin AV, Grossmann R, Maria-Peon MT, Roa J, Tena-Sempere M & Klein S 2006 Novel expression and functional role of ghrelin in chicken ovary. *Molecular and Cellular Endocrinology* **257–258** 15–25. (doi:10.1016/j.mce.2006.06.004)
- Small BC, Quiniou SM & Kaiya H 2009 Sequence, genomic organization and expression of two channel catfish, *Ictalurus punctatus*, ghrelin receptors. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **154** 451–464. (doi:10.1016/j.cbpa.2009.07.027)
- Sun Y, Garcia JM & Smith RG 2007 Ghrelin and growth hormone secretagogue receptor expression in mice during aging. *Endocrinology* **148** 1323–1329. (doi:10.1210/en.2006-0782)
- Tachibana T, Tanaka M & Kaiya H 2011 Central injection of des-acyl chicken ghrelin does not affect food intake in chicks. *General and Comparative Endocrinology* **171** 183–188. (doi:10.1016/j.ygcen.2011.01.008)
- Takahashi K, Furukawa C, Takano A, Ishikawa N, Kato T, Hayama S, Suzuki C, Yasui W, Inai K, Sone S *et al.* 2006 The neuromedin U-growth hormone secretagogue receptor 1b/neurotensin receptor 1 oncogenic signaling pathway as a therapeutic target for lung cancer. *Cancer Research* **66** 9408–9419. (doi:10.1158/0008-5472.CAN-06-1349)
- Tanaka M, Miyazaki T, Yamamoto I, Nakai N, Ohta Y, Tsushima N, Wakita M & Shimada K 2003 Molecular characterization of chicken growth hormone secretagogue receptor gene. *General and Comparative Endocrinology* **134** 198–202. (doi:10.1016/S0016-6480(03)00247-8)
- Tang XL, Wang Y, Li DL, Luo J & Liu MY 2012 Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. *Acta Pharmacologica Sinica* **33** 363–371. (doi:10.1038/aps.2011.210)
- Tannenbaum GS & Bowers CY 2001 Interactions of growth hormone secretagogues and growth hormone-releasing hormone/somatostatin. *Endocrine* **14** 21–27. (doi:10.1385/ENDO:14:1:021)
- Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, Date Y, Mondal MS, Shimbara T, Kawagoe T *et al.* 2006 Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* **147** 2306–2314. (doi:10.1210/en.2005-1357)
- Ueberberg B, Unger N, Saeger W, Mann K & Petersenn S 2009 Expression of ghrelin and its receptor in human tissues. *Hormone and Metabolic Research* **1** 814–821. (doi:10.1055/s-0029-1233462)
- Verhulst PJ & Depoortere I 2012 Ghrelin's second life: from appetite stimulator to glucose regulator. *World Journal of Gastroenterology* **18** 3183–3195. (doi:10.3748/wjg.v18.i25.3183)
- Wettschureck N, Moers A, Wallenwei B, Parlow AF, Maser-Gluth C & Offermanns S 2005 Loss of Gq/11 family G proteins in the nervous system causes pituitary somatotroph hypoplasia and dwarfism in mice. *Molecular and Cellular Biology* **25** 1942–1948. (doi:10.1128/MCB.25.5.1942-1948.2005)
- Yamamoto I, Yoshimura Y, Tsukada A, Kansaku N, Tsushima N & Tanaka M 2008 Predominant expression of growth hormone secretagogue receptor in the caudal lobe of chicken pituitary and parallel development increase in expression of pituitary GH and proventriculus ghrelin mRNA. *Animal Science Journal* **79** 116–121. (doi:10.1111/j.1740-0929.2007.00506.x)
- Yang J, Brown MS, Liang G, Grishin NV & Goldstein JL 2008 Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* **132** 387–396. (doi:10.1016/j.cell.2008.01.017)
- Zaccone D, Sengar M, Lauriano ER, Pergolizzi S, Macri' F, Salpietro L, Favaloro A, Satora L, Dabrowski K & Zaccone G 2012 Morphology and innervation of the teleost physostome swim bladders and their functional evolution in non-teleostean lineages. *Acta Histochemica* **114** 763–772. (doi:10.1016/j.acthis.2012.01.003)
- Zigman JM, Jones JE, Lee CE, Saper CB & Elmquist JK 2006 Expression of ghrelin receptor mRNA in the rat and the mouse brain. *Journal of Comparative Neurology* **494** 528–548. (doi:10.1002/cne.20823)

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