Chapter 10
Fatty Acids Receptors

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Abstract In the past decade, a strategy to deorphanize G protein-coupled receptors (GPCRs) has identified a series of receptors for free fatty acids (FFAs) that play significant roles in nutrition regulation. In this free fatty acid receptor family, FFAR1 (GPR40) and FFAR4 (GPR120) are activated by medium- and long-chain FFAs. FFAR1 regulates insulin secretion in pancreatic β-cells, whereas FFAR4 promotes the secretion of glucagon-like peptide-1 (GLP-1) in the intestine and also act as the lipid sensor in the adipose tissue to sense dietary fat and control energy balance. In this chapter, we discuss recent advances in the identification of ligands and the pharmacological characterization of FFAR1 and FFAR4, and we present a summary of the current understanding of their physiological roles and potential as drug targets.

Keywords GPCR • Fatty acid receptor • FFAR1 • FFAR4 • GPR40 • GPR120

10.1 Introduction

Free fatty acids (FFAs) are not only essential dietary nutrients but they also act as signaling molecules in various physiological functions. The nuclear receptors peroxisome proliferator-activated receptors (PPARs) and fatty acid-binding proteins (FABPs) are known to act as 'sensors' of FFAs. They maintain homeostasis under

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T. Yokomizo, M. Murakami (eds.), *Bioactive Lipid Mediators*,
DOI 10.1007/978-4-431-55669-5_10

physiological and pathophysiological conditions by coordinating the expression of proteins involved in lipid uptake, synthesis, transport, storage, degradation, and elimination [5]. However, these mechanisms could not explain all biological effects of FFAs. Some effects were thought to be mediated by other mechanisms such as signaling through cell-surface receptors [28, 40]. In the last decade, a strategy to deorphanize G protein-coupled receptors (GPCRs) has identified a series of such receptors for FFAs that have significant roles in nutrition regulation (Table 10.1). Among FFARs, FFAR2 (GPR43) and FFAR3 (GPR41) are activated by short-chain FFAs (SCFAs) such as acetate, propionate, and butyrate. On the other hand, GPR84 is activated by medium-chain fatty acids, and FFAR1 (GPR40) and FFAR4 (GPR120) are activated by medium- and long-chain saturated and unsaturated FFAs. The importance of the characterization of these GPCRs is emphasized by the fact that 30 % of all prescription drugs target GPCRs, and many groups have reported that these FFARs are expressed in the gastrointestinal tract and have several important roles involved in energy homeostasis. These FFARs are also widely conserved among vertebrates, which suggests that they have common important physiological functions. Therefore, FFARs have received considerable attention as potential therapeutic targets for metabolic disorders. In this chapter, we focus on recent advances in our understanding of FFARs, especially FFAR1 and FFAR4, and their roles in energy homeostasis.

10.2 FFAR1

10.2.1 Ligand and Tissue Distribution

FFAR1 is activated by medium- and long-chain saturated and unsaturated FFAs, as reported by three independent groups almost simultaneously [1, 24, 27]. A variety of FFAs has been found to act as agonists of FFAR1 in the micromolar concentration range, with eicosatrienoic acid being the most potent. FFAR1 is enriched 2- to 100-fold in pancreatic islets as compared with the whole pancreas. FFAR1 is also expressed in the intestine. FFAR2 and FFAR3, on the other hand, are both activated by short-chain FFAs, such as formate, acetate, propionate, butyrate, and pentanoate [7, 8, 13]. FFAR3 is activated equally by propionate, butyrate, and pentanoate, whereas FFAR2 prefers propionate over the other short-chain FFAs [7, 8]. Short-chain fatty acids activate FFAR2 or FFAR3 in a relatively high submillimolar concentration range. FFAR2 and FFAR3 are expressed in the adipose tissue and sympathetic ganglions, respectively [26]. There are also several reports suggesting that fermentation end products, especially short-chain fatty acids produced by gut microbiota, affect inflammation via FFARs [29, 38].

Table 10.1 Free fatty acids receptors family

Nomenclature	FFAR1 GPR40	FFAR2 GPR43	FFAR3 GPR41	FFAR4 GPR120	 GPR84
Agonist(FFA)	Medium-long	Short Chain C3∼C4∼C2	Short Chain C3>C4>>C2	Medium-long	Medium- 2-OH- or 3-OH MCFA
(Other)	TAK-875,Thiazolidinedione			NCG21	6-OAU
G protein coupling	Gq/11	Gq/11,Gi/o	Gi/o	Gq/11	—
Gene/chromosomal	GPR40	GPR43	GPR41	GPR120	GPR84
Localization	19q13,1	19q13.1	19q13.1	10q23.33	12q13.13
Protein(human)	NP_005294, 300a.a	NP_005297, 330a.a	NP_005295, 346a.a	NP_859529, 377a.a	NP_065103 396a.a.
Expression	Pancreatic β-cell intestine	Adipose tissue intestine	Adipose tissue Sympathetic ganglions	Colon/Adipose tissue	Spleen(B cell & T cell)
Physiological role	Insulin secretion	Lipid and energy metabolism anti-inflammatory effect	Energy regulation	GLP-1 secretion	IL-12 p40

Table modified from references [32].

10.2.2 Genomic Structure and Evolution

FFAR1, FFAR2, FFAR3, and GPR42, which is thought to be a pseudogene, are all GPCRs of the rhodopsin family located within a gene cluster on the human 19q13 chromosome. GPR42 only exists in the family *Hominidae* and cannot be detected in species below gibbons. The members of this subfamily share approximately 30–40 % identity, with the exception that human GPR42 (hGPR42) differs from human FFAR3 (hFFAR3) at only six amino acid positions [3]. These findings suggest that hGPR42 arose as the result of a gene duplication of hFFAR3 that occurred after the gibbons branched off from the superfamily *Hominoidea* [3]. Recent advances in genomic analysis of various species have revealed that FFAR1, FFAR2, and FFAR3 are widely conserved throughout vertebrates from fishes to mammals (with the exception of birds). In amphibians, reptiles, and mammals, FFAR1, FFAR2, and FFAR3 create a family of genes in tandem sequence with shared synteny (Fig. 10.1). However, similar genomic structures cannot be found in birds, at least among genomically analyzed species such as pigeons and chickens. On the other hand, we have found multiple clusters of these genes existing in *Teleostei*, which suggests that multiplication of these genes occurred after the divergence of amphibians. We have also found that only one homologous gene exists in cartilaginous fishes. To date, the function of FFARs have only been investigated in mammals, and we lack information about their expression and physiological function in other species. Thus, the connection between these receptors and physiological functions are not fully understood and should become a matter of great interest in the upcoming years.

10.2.3 Signal Transduction

In Chinese hamster ovary (CHO) cells, exogenously expressed FFAR1 is coupled to the formation of inositol 1,4,5-trisphosphate, intracellular Ca^{2+} mobilization, and the activation of extracellular signal-regulated kinase (ERK) 1/2 [24]. These results suggest that FFAR1 is coupled to Gαq and/or $G_{i/o}$ protein. Fujiwara et al. showed, in rat islet β-cells, that oleic acid (OA) interacts with FFAR1 to increase intracellular Ca^{2+}, via the phospholipase (PL) C- and L-type Ca^{2+} channel-mediated pathway, which links to insulin release [10]. Feng et al. showed that linolenic acid reduces the voltage-gated K^+ current in rat pancreatic β-cells through the FFAR1-mediated regulation of cAMP levels and protein kinase A activity; the reduction in K^+ current leads in turn to enhanced β-cell excitability and insulin secretion [9].

10.2.4 Protein Structure and New Chemicals

The first report identifying a series of FFAR1 agonists based on 3-(4-[*N*-alkyl]aminophenyl)propanoic acid was by Garrido et al. [12]. In particular, the physiological and pharmacological properties of GW9508 have been studied in detail, as it has potential as an agonist for not only FFAR1 but also FFAR4 (Fig. 10.2). Furthermore,

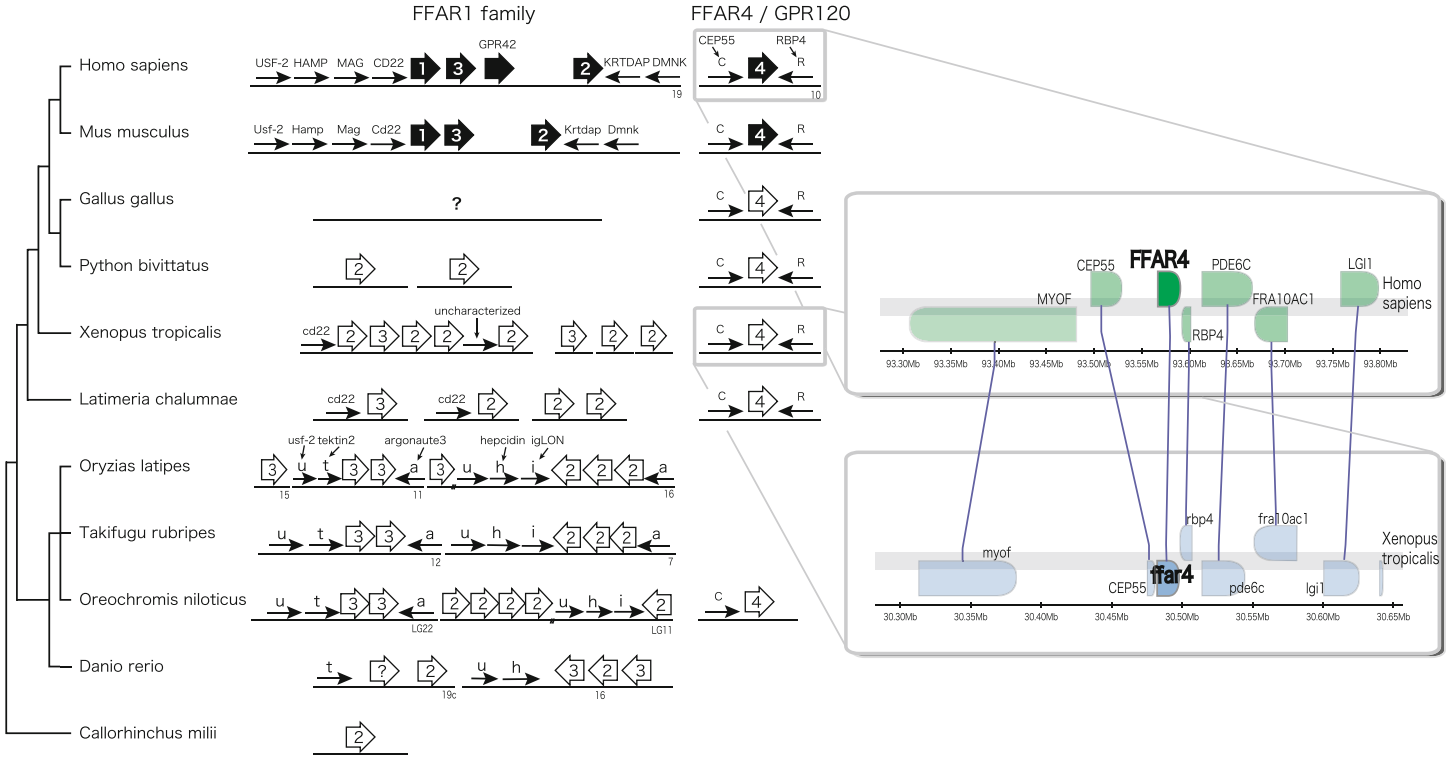

Fig. 10.1 Schematic representation of genomic structures around free fatty acids receptors (FFAR)1–4 of multiple animal species, and synteny analysis of the FFAR4 locus between *Homo sapiens* and *Xenopus tropicalis* genomes. The homology genes of FFAR1 and FFAR4 are compared and aligned in each species. *Black* or *white boxes* indicate genes that have been studied or not, respectively. Synteny analysis using Synteny Database [4] reveals syntenic conservation of many neighbor genes around FFAR4 between human and *Xenopus* (cluster ID#1424337 according to the Synteny Database)

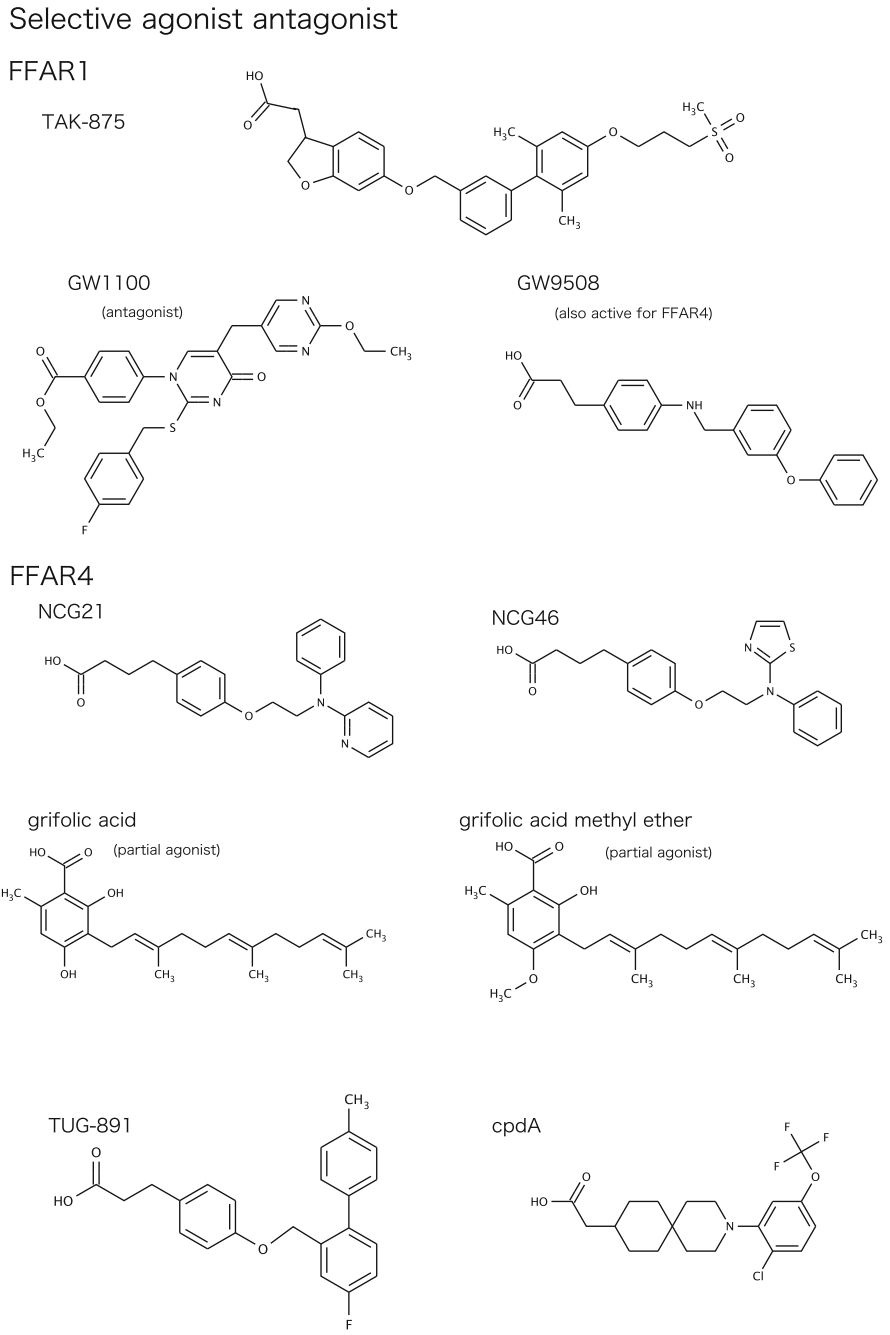

Fig. 10.2 Chemical structures of FFAR1 and FFAR4 ligands

a synthetic FFAR1 antagonist, GW1100, has been identified and its antagonistic activities were examined via in both in vitro and in vivo studies [1, 19, 22, 53]. The antidiabetic thiazolidinediones troglitazone and rosiglitazone, and the experimental anti-obesity compound MEDICA16, also activate FFAR1 [2, 16]. Very recently, the X-ray crystallography of co-crystals of a novel compound, TAK-875 (fasiflifam), and FFAR1 was performed, and the state of the ligand binding to the receptor was revealed [42]. It was reported that binding sites other than those predicted in recent models exist and that TAK-875 could act as a partial agonist or an allosteric ligand. In the same paper, a mass spectrometry-based ligand-binding assay system for FFAR1 was also established, and the FFAR–ligand interaction has been studied in great detail.

10.2.5 Physiological Roles of FFAR1

Free fatty acids have so far been known to exert versatile effects on pancreatic β-cells. Chronic exposure to high levels of FFAs results in the impairment of β-cell function and secretory capacity, whereas acute administration of FFAs stimulates insulin release. FFAs are considered to be important for maintaining basal insulin secretion as well as increasing glucose-stimulated insulin secretion when fasting [1, 7, 8, 13, 43, 44], but the mechanisms of these phenomena have not been explained. Itoh et al. showed that long-chain free fatty acids amplified glucose-stimulated insulin secretion from pancreatic β-cells via activation of FFAR1 [24]. Because inhibition of FFAR1 expression with small interfering RNA (siRNA) resulted in quenching of FFA-stimulated insulin secretion, FFAR was presumed to be involved in this pathway. In contrast to the decrease in FFA-stimulated insulin secretion observed in FFAR1-deficient β-cells, FFAR1-deficient mice actually exhibited resistance to obesity-induced hyperinsulinemia, hepatic steatosis, hypertriglyceridemia, increased hepatic glucose output, hyperglycemia, and glucose intolerance. Steneberg et al. have indicated that both acute and chronic effects of FFAs were mediated by FFAR1 [44]. In contrast, overexpression of FFAR1 in β-cells by methods via the mouse Ipf1/Pdx1 promoter impaired β-cell function and resulted in hyperinsulinemia and diabetes [44]. Moreover, FFAR1 was found to regulate glucose-stimulated insulin secretion by overexpressing FFAR1 under the control of mouse insulin II promoter [33]. On the basis of these studies, we speculate that FFAR1 is involved in an essential pathway that connects obesity and type 2 diabetes.

Furthermore, there have been several studies reporting genomic polymorphisms in human FFAR1. One type of polymorphisms, D175N, has the same EC_{50} but lower maximum response compared to the wild-type receptor. This polymorphism, however, has no relevance to changes in insulin secretion [15]. Another polymorphism, R211H, shows no difference in primary response, but results from laboratory data comparison suggests its involvement in insulin secretion [34]. Moreover, β-cell response to FFAs is quenched in the polymorphism G180S because of impaired mechanisms in increasing intracellular Ca^{2+} concentration [52].

These results have raised a great deal of interest in FFAR1 as a potential target for novel drugs in metabolic diseases such as type 2 diabetes. Various experimental models have identified chemical compounds that display agonistic or antagonistic activity, and their physiological and pharmacological functions are being examined. One such compound was TAK-875 (fasiglifam, mentioned earlier), an orally available, potent, and selective agonist of FFAR1 [39]. This agent was tested in a phase III clinical trial for the potential treatment of type 2 diabetes mellitus, but the trial was cancelled because of an undesired side effect. Another FFAR1 agonist, JTT-851, has completed phase II clinical trials and is anticipated to become the first therapeutic drug to target FFAR1.

10.3 FFAR4

10.3.1 Ligand and Tissue Distribution

Using a receptor internalization assay [11], medium- to long-chain FFAs were identified as endogenous ligands of FFAR4. Saturated FFAs (C14-18) and unsaturated FFAs (C16-22) activate FFAR4. Although some have claimed that FFAR4 was a selective receptor for ω-3 polyunsaturated fatty acids (PUFAs), it is now widely accepted that both ω-3 and ω-6 PUFAs act as agonists. A variety of PUFAs, regardless of ω-3 or ω-6 species, can activate FFAR4 in the micromolar concentration range [18]. The ligand profiles for FFAR4 are similar to those for FFAR1; however, the amino acid homology between FFAR4 and FFAR1 is only 10 %.

10.3.2 Genomic Structure and Evolution

FFAR1 and FFAR4 have no homology in structure, although some ligands activate both receptors. FFAR4 has been experimentally proved to function as a receptor in only mammals. Nonetheless, the FFAR4 gene is conserved in vertebrates from *Coelacanthiformes* to mammals, and the neighboring genomic structures are also quite similar, as shown in the comparison of the human and *Silurana tropicalis* genomes (Fig. 10.1). However, differing from FFAR1, FFAR2, and FFAR3, which prevail throughout vertebrates, the FFAR4 gene cannot be seen in teleost fish, with an exception of the family *Cichlidae*. Sequences similar to FFAR4 are detected in *Cichlidae* genomes, but the reason for this exception is unknown. Multiplication of genes such as those in FFAR1, FFAR2, and FFAR3 are also not observed in FFAR4. Comparing the FFAR4 orthologues including *Cichlidae*, we found that the chief amino acid sequences are well conserved among species. The amino acid residue equivalent to human R99 (described later in this chapter), which is important for the interaction of FFAR4 and FFAs, is conserved in all species, strongly suggesting that the function is conserved as well (Fig. 10.3).

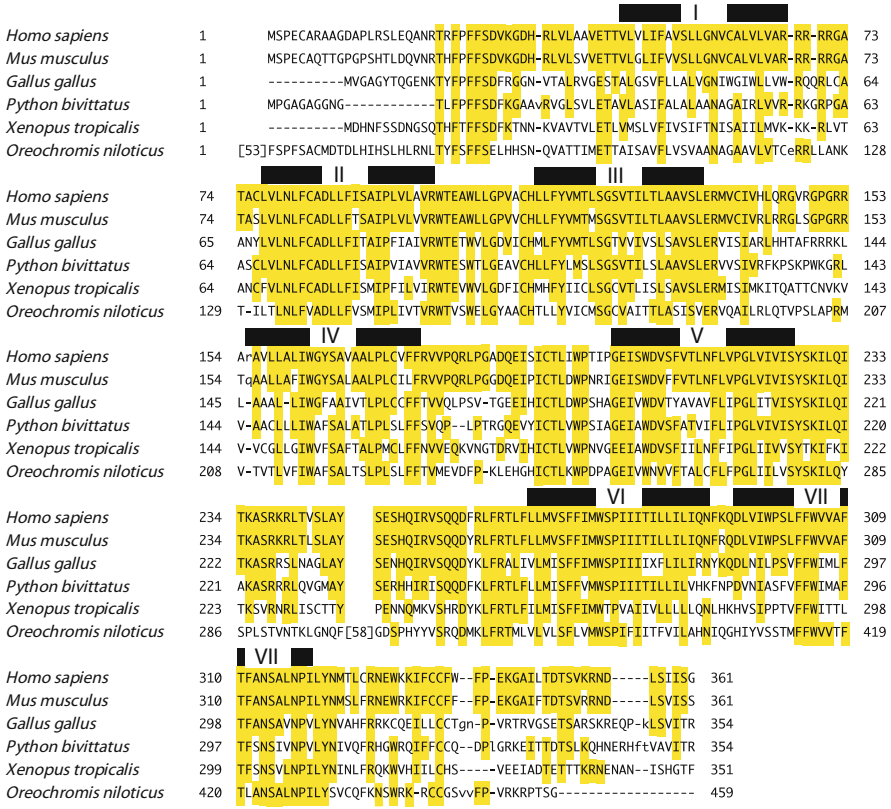

Fig. 10.3 Sequence alignment of FFAR4 in multiple animal species. The alignment is obtained by multiple sequence alignment for six homology protein sequences. Amino acid sequences corresponding to FFAR4 from different animal species were aligned using the NCBI COBALT algorithm [37]. Transmembrane ™ helix regions are shown between *black bars*. Highly conserved residues are highlighted by *yellow*

10.3.3 Signal Transduction

Both PUFAs and synthetic ligands induced a rise in cytosolic free Ca^{2+} in FFAR4-overexpressing HEK293 cells, suggesting that FFAR4 is coupled with the Gαq protein family. Recently, Shah et al. showed that PUFA-induced depolarization induced by the monovalent cation-specific transient receptor potential channel type M5 (TRPM5) is related to intracellular Ca^{2+} rise as well as CCK secretion from STC-1 cells, suggesting that TRPM5 plays a crucial role in FFAR4 signaling in STC-1 cells [41]. Oh et al. showed that a FFAR4 agonist exerts anti-inflammatory effects through β-arrestin 2 signaling in monocytic RAW 264.7 cells and primary macrophages [36]. FFAR4 can also induce the activation of ERK1/2 under certain conditions and activation of PI3-kinase and the serine/threonine protein kinase Akt in FFAR4-expressing cells [17, 25].

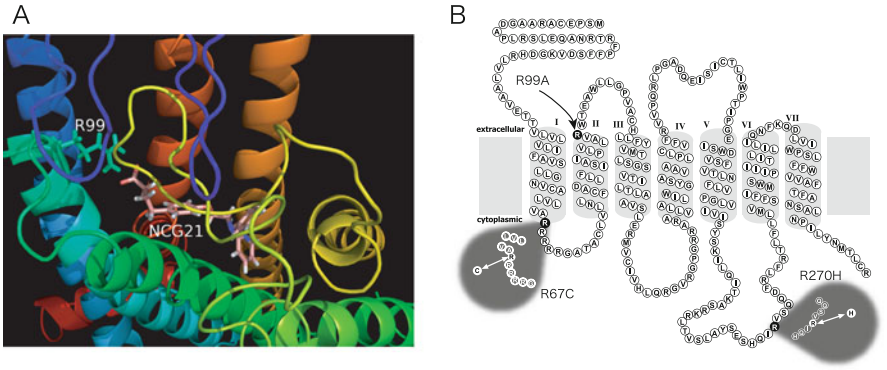

Fig. 10.4 Single-nucleotide polymorphisms (SNPs) in human FFAR4 and homology model of FFAR4. (**a**) Representation of secondary structure of FFAR4. Locus of its SNPs (R67C, R270H) and an interaction site (R99) are marked. (**b**) FFAR4 homology model docked with NCG21

10.3.4 Structure–Activity Relationships of FFAR4 Ligands

Because the three-dimensional structure of FFAR4 has not yet been elucidated by X-ray crystallography, structure–activity relationship studies are being conducted by combining site-directed mutagenesis and homology modeling. The results of the site-directed mutagenesis showed that the R99 residue is significant for the ligand binding for FFAR4, and the amino acid sequences around R99 are well conserved in aforementioned orthologues [47] (Figs. 10.3 and 10.4a). The calculation of the docking simulation and homology model of FFAR4 revealed significant correlation between the calculated value of the hydrogen bond energy and ligand-induced activity in many compounds, which led us to predict the activity of novel compounds [47, 49]. To identify other natural ligands of FFAR4, we screened and identified a selective partial agonist among a series of natural compounds derived from fruiting bodies of *Albatrellus ovinus* [17]. Depending on the experimental conditions, this compound is also useful as an antagonist selective for FFAR4. In addition, based on the structure of the PPARγ agonist thiazolidinediones, we synthesized a series of compounds containing carboxylic acids and developed a selective agonist using a homology model of FFAR4 [48] (Fig. 10.2). Hudson et al. have also reported the synthesis of compounds selective for FFAR4 [20], and many patents of compounds have been claimed [14]. The structure–activity relationship studies combining site-directed mutagenesis and homology modeling of FFAR4 showed that hydrophobic amino acid residues facing the ligand-binding pocket play an important role in the binding of FFAR4 ligand [21]. These compounds might be useful tools to monitor the physiological effects of FFAR4, and they might be potentially useful in the development of novel drug candidates for the treatment of type 2 diabetes, obesity, and metabolic diseases.

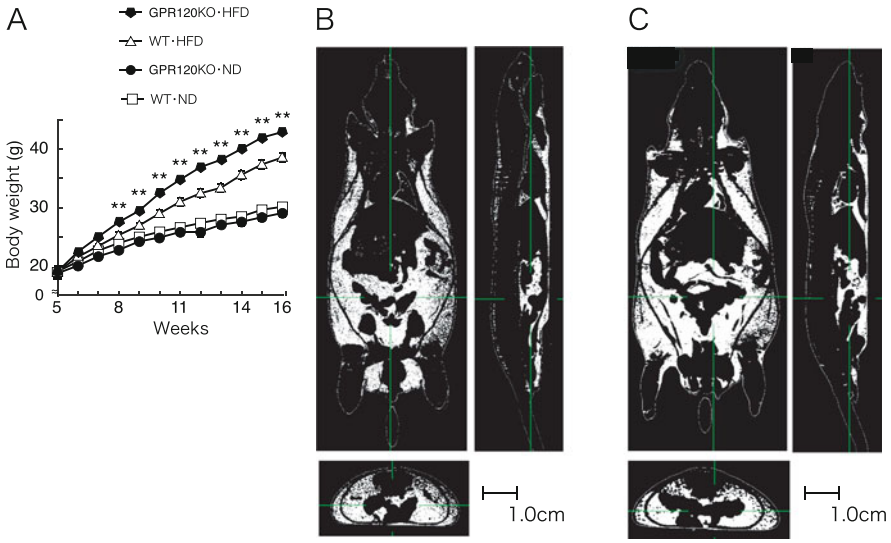

Fig. 10.5 Obesity in FFAR4-deficient mice fed a high-fat diet. (**a**) Body weight changes of wild-type and FFAR4-deficient mice fed a normal diet (ND) or a high-fat diet (HFD). All data represent mean + SEM. $^{**}P < 0.01$ versus the corresponding wild-type value. (**b**, **c**) Computed tomography images of fat accumulation in wild-type (**b**) and FFAR4-deficient (**c**) male mice fed a high-fat diet. Fat depots are demarcated for illustration

10.3.5 Physiological Roles of FFAR4

We recently reported that dysfunctional FFAR4 led to obesity in both mice and humans [23]. We found that FFAR4-deficient mice fed a high-fat diet developed obesity, glucose intolerance, and fatty liver along with decreased adipocyte differentiation and lipogenesis and enhanced hepatic lipogenesis (Fig. 10.5). Insulin resistance in these mice was associated with reduced insulin signaling and enhanced inflammation in adipose tissue. FFAR4 exon sequencing in obese subjects revealed a deleterious nonsynonymous mutation (R270H) that inhibited FFAR4 signaling activity (Fig. 10.4b). Furthermore, the R270H variant increases the risk of obesity in European populations. Overall, this study demonstrates that the lipid sensor FFAR4 has a key role in sensing dietary fat and, therefore, in the control of energy balance in both humans and rodents.

Endogenous expression of FFAR4 has been identified in the intestine of humans and mice. Our previous study showed that FFAR4-expressing cells were located in the GLP-1-expressing enteroendocrine cells in the large intestine [18, 31]. Furthermore, the enteroendocrine cell line STC-1 also expressed FFAR4 endogenously, and PUFA or synthetic ligand stimulation induced the secretion of GLP-1 and cholecystokinin (CCK) as well as the $[Ca^{2+}]_i$ response [50]. These studies led us to speculate the physiological function of FFAR4 in incretin secretion in vivo.

FFAR4 is also expressed in other cells and tissues. Oh et al. found endogenous expression of FFAR4 in monocytic RAW 264.7 cells and in primary pro-inflammatory, M1-like macrophages. Matsumura et al. found the expression of FFAR4 in taste buds [30]. In the lung, we found that FFAR4 protein was colocalized with the Clara cell 10-kDa protein, a marker of Clara cells [31]. Further studies are needed to reveal the physiological function of FFAR4 in the lung.

Taneera et al. performed a systems genomics approach to identify genes for type 2 diabetes, and FFAR4 was ranked among the top 20 possibly associated genes [51]. In this report, FFAR4 expression in human islets was positively correlated with insulin secretion and insulin content and with lower HbA1c. Although inconsistent with previous reports that FFAR1 is dominantly detected in mouse pancreatic islets [33], these data suggest that FFAR4 can protect pancreatic islets from lipotoxicity in humans.

Recently, it has become clear that FFAR4 is expressed in the pancreas and contributes to glucagon secretion [46]. According to a report by Oh et al., continued administration of an agonist selective for FFAR4 improved glucose tolerance and insulin sensitivity in mice fed a high-fat diet [35]. However, they attributed their results to a macrophage-mediated pathway and did not mention its relationship to obesity; this is in disagreement with our results.

From the analyses of human and mice described here, there is no doubt that FFAR4 is strongly involved in diet-induced obesity and acts as a lipid sensor that maintains the balance of energy metabolism by controlling lipid biosynthesis [23]. Further investigation is anticipated to develop therapeutic drugs targeting FFAR4 for the treatment of obesity-related metabolic disorders.

10.4 Conclusion

Because of the low binding affinity of FFAs to FFARs, there was skepticism toward FFARs when originally discovered. However, the experimental facts described here confirmed that FFAs were indeed ligands for FFARs; thus, the names of the fatty acid receptor family, which were originally the numbers of the orphan receptors (GPR40, GPR120, etc.), are now officially changed to FFAR1, FFAR4, etc, respectively. [6, 45]. The ligands for some nutrient-sensing GPCRs bind with lower affinity (in the micromolar or millimolar range) than that of classic high-affinity ligands, such as hormones or growth factors, for their receptors. The fatty acid receptor family is considered to be a group of sensor molecules that detect FFAs of various lengths and structures as natural ligands with binding constants comparable with their vivo concentrations. Among the FFARs, FFAR1 and FFAR4 are considered to be potential drug targets for the treatment of metabolic diseases such as type 2 diabetes, because their physiological functions are related to energy homeostasis. Further analysis of FFARs may also be important to better understand the nutrient-sensing process and to develop novel therapeutic compounds to treat metabolic diseases.

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