Domestic Animal Endocrinology 47 (2014) 108–118

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

Identification and characterization of the free fatty acid receptor 2 (FFA2) and a novel functional FFA2-like receptor (FFA2L) for short-chain fatty acids in pigs: Evidence for the existence of a duplicated FFA2 gene (*FFA2L*) in some mammalian species

J. Zhang, S. Cheng, Y. Wang*, X. Yu, J. Li*

Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu, 610065, PR China

ARTICLE INFO

Article history:
Received 10 August 2013
Received in revised form 21 October 2013
Accepted 25 October 2013

Keywords:
Pig
Short-chain fatty acids
FFA2
FFA2-like receptor

ABSTRACT

Free fatty acid receptor 2 (FFA2, also called GPR43) is reported to play a critical role in mediating the actions of short-chain fatty acids (SCFAs) in humans and mice. However, little is known about the structure, functionality, and tissue expression of *FFA2* in other mammalian species, including pigs. In the present study, the full-length cDNAs of *FFA2* (*pFFA2*) and a novel *FFA2*-like gene (named *pFFA2L*) were cloned from pig intestines by reverse transcription PCR. Both cloned *pFFA2* and *pFFA2L* are predicted to encode 2 receptors of remarkable structural similarity and share high amino acid sequence identities with FFA2 from other mammalian species. Interestingly, the novel *FFA2L* could also be identified in 9 other mammalian species, suggesting that *FFA2L* was likely duplicated from *FFA2* in the last common ancestor of these species. With the use of a pGL4-SRE-luciferase reporter assay, we demonstrated that pFFA2 expressed in human embryonic kidney 293 cells could be activated by acetate, propionate, and butyrate equipotently, whereas pFFA2L could be activated only by acetate and propionate, indicating that both pFFA2 and pFFA2L are functional receptors for SCFAs with nonidentical pharmacologic properties. Reverse transcription PCR found that *pFFA2* mRNA was widely expressed in nearly all tissues examined, including adipose tissue and gastrointestinal (GI) tract, whereas *pFFA2L* expression was mainly restricted to the GI tract. Taken together, our findings raise a novel concept that the actions of SCFAs are likely mediated by 2 FFA2s (FFA2 and FFA2L) in target tissues of some mammalian species, such as the GI tract of pigs.

1. Introduction

It is well documented that short-chain fatty acids (SCFAs, consisting of 1–6 carbon atoms), such as acetate, propionate, and butyrate, are produced in the large intestine by bacterial fermentation of ingested carbohydrates and dietary fibers in mammals [1,2]. Originally, these SCFAs have been viewed as the nutrients and transported into the bloodstream; however, evidence has grown during the past decade that SCFAs can function as the key signaling molecules to regulate many physiological and pathologic processes, including gastrointestinal tract (GI) functions, inflammation, and obesity [2–4]. It is reported that in mammals, SCFA actions are mediated by the 2 G protein–coupled receptors (GPCRs): free fatty acid receptor 2 (FFA2, also called GPR43) and free fatty acid receptor 3 (FFA3; also called GPR41), both of which have been found to be localized on the same chromosome and share 43% amino acid sequence identity [1]. Free fatty acid receptor 2 can bind to acetate (C2), propionate (C3), and butyrate (C4) within micromolar affinity, whereas FFA3 preferentially binds to

* Corresponding author. Tel.: +86 28 8541 5025; fax: +86 28 8541 4886.
E-mail addresses: cdwyjhk@gmail.com (Y. Wang), lijuanhk@gmail.com (J. Li).

0739-7240/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.domaniend.2013.10.004

propionate, valerate (C5), and butyrate with relatively higher affinities than the other SCFAs, including acetate (C2) and hexanoate (C6) [5–7]. Among the 2 receptors, FFA2 has attracted much more attention as the promising drug target for treatment of diseases such as inflammation, obesity, and cancer [2]. Free fatty acid receptor 2 is reported to be functionally coupled to both $G\alpha_{i/o}$ and $G\alpha_q$ proteins and capable of initiating multiple signaling cascades, including inhibition of intracellular cyclic adenosine monophosphate levels, elevation of intracellular calcium levels, and activation of mitogen-activated protein kinase (MAPK) signaling pathway on SCFA binding [6,8]. In agreement with the diverse actions of SCFAs in mammals, *FFA2* has been found to be expressed in a variety of tissues, including immune cells, adipose tissue, muscle, heart, distal ileum, and colon [2]. In mouse adipose tissue, FFA2 expression is reported to be associated with SCFA-induced adipogenesis and SCFA-inhibited lipolysis [9–11]. In the GI tract, FFA2 has been found to be expressed throughout the gut and directly involved in SCFA-regulated GI tract functions, such as stimulation of intestinal peptide YY and glucagon-like peptide 1 secretion [12–15]. Moreover, FFA2 expression in the GI tract has been suggested to be involved in the onset and progression of colon cancer through its antiproliferative and apoptotic effects on target cells [16]. All the findings emphasize the critical roles of FFA2 in mediating the actions of SCFAs in mammals.

In contrast to the extensive study on the structure–activity relationships, signaling property, and physiological roles of FFA2 in humans, rats, and mice [1,2], the structure, functionality, tissue expression, and physiological roles of *FFA2* remains largely unknown in other mammalian species [17,18], although *FFA2* recently was reported to be expressed in pig tissues. In the present study, we targeted pigs as our experimental animal, given its biomedical and breeding importance, and cloned *FFA2* from pig intestine and characterized its tissue expression and functionality. Interestingly, in addition to the identification of the *FFA2* in pigs, a novel FFA2-like receptor (named *FFA2L*) was also identified in pigs and 9 other mammalian species, including elephants, pandas, and bushbabies in this study. Our findings will facilitate the better understanding of the physiological roles played by FFA2, FFA2L, and their ligands SCFAs in mammals, such as their roles in regulation of adipogenesis and GI tract functions. Meanwhile, the identification of a novel *FFA2*-like gene in pigs and other mammalian species raises a potential novel concept that the biological actions of SCFAs may be mediated by 3 specific receptors, FFA2, FFA2L, and FFA3, in some mammalian species, such as pigs.

2. Materials and methods

2.1. Chemicals and hormones

Three SCFAs, including sodium acetate (C2), sodium propionate (C3), sodium butyrate (C4), and a long-chain fatty acid docosahexaenoic acid (DHA; C22:6 n-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Drugs were freshly prepared before use and dissolved in distilled water or dimethylsulfoxide (for DHA). Restriction enzymes were obtained from TaKaRa Biotechnology (Dalian, China). The other experimental reagents were purchased from Sigma-Aldrich.

2.2. Total RNA extraction

Adult pigs were killed, and different tissues, including heart, kidney, liver, lung, muscle, ovary, testis, pituitary, spleen, tongue, subcutaneous adipose tissue, various brain regions (cerebrum, cerebellum, and hypothalamus), and GI tract (stomach, duodenum, ileum, cecum, proximal colon, distal colon, and rectum) were collected for total RNA extraction. Total RNA was extracted from tissues with RNAzol Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions and dissolved in diethylpyrocarbonate-treated H_2O. The experiments were performed according to the guidelines provided by the Animal Ethics Committee of Sichuan University.

2.3. Reverse transcription and PCR

Reverse transcription (RT) was performed at 42°C for 2 h in a total volume of 10 μL that consisted of 2 μg of total RNA from different tissues, 1× single strand buffer, 0.5 m*M* each deoxynucleotide triphosphate, 0.5 μg oligo-deoxythymidine, and 100 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). All negative controls were performed under the same condition without reverse transcriptase added in the reaction mix.

According to our previously established methods [19], RT-PCR assays were performed to examine the mRNA expression of *FFA2* and *FFA2L* in adult pig tissues. DNase I–pretreated total RNA was reverse-transcribed in the presence or absence of reverse transcriptase, as described in our previous study. For the glyceraldehyde phosphate dehydrogenase gene, PCR was conducted under the following conditions: 95°C for 30 s, 26 cycles of amplification (95°C for 30 s, 56°C for 30 s, 72°C for 30 s), and a 20-min final extension at 72°C. For the *FFA2* (or *FFA2L*) gene, 95°C for 30 s of denaturation, 33 cycles of amplification (30 s at 95°C, 30 s at 58°C, 40 s at 72°C), and then followed by a 20-min extension at 72°C. The primers used are listed in Table 1. The PCR products were visualized on a UV-transilluminator (Bio-Rad Laboratories, Inc, Hercules, CA, USA) after electrophoresis on 2% agarose gel stained with ethidium bromide. To confirm the specificity of PCR reaction, the identity of PCR products were verified by sequencing.

2.4. Cloning the full-length cDNAs of FFA2 and FFA2L from pig intestine and construction of their expression plasmids

To obtain the full-length cDNA of *FFA2* and *FFA2L*, 2 gene-specific primers that flank the start and stop codons (with a restriction enzyme recognition site added at the 5′ end) were designed on the basis of the sequence information of pig genome (http://www.ensembl.org/Sus_scrofa) and predicted sequences of *FFA2* and *FFA2L* (Accession nos. XM_003127046 and XM_003127052) (Table 1). The cDNAs covering the complete open reading frame (ORF) of *FFA2* and *FFA2L* were amplified from adult pig intestine with the use of high-fidelity *Taq* DNA polymerase (Toyobo Co, Ltd,

Table 1
Primers used for cloning and RT-PCR assay.

Gene	Sense/antisense	Primer sequence (5′–3′)	Size (bp)
Primers for cloning the full-length cDNAs[a]			
pFFA2	Sense	CGCGGATCCATCCTCTTCCAGAACAGTC	1068
	Antisense	CCGGAATTCCACCCAGACACCACCCTGA	
pFFA2L	Sense	CGGGGTACCCTTTGTCTCACCAGGAAG	1102
	Antisense	CCGGAATTCTAGAGGAGAACTCACTCTG	
Primers for RT-PCR assay			
pFFA2	Sense	GTACCTGCCTGGGATCGTCT	359
	Antisense	TGACCACCATGGGGATGAAG	
pFFA2L	Sense	GAGAGCCGGAAGCGCTGGA	448
	Antisense	CCGGAATTCTAGAGGAGAACTCACTCTG	
pGAPDH	Sense	ACCACAGTCCATGCCATCAC	452
	Antisense	TCCACCACCCTGTTGCTGTA	

Abbreviations: pFFA2, pig free fatty acid receptor 2; pFFA2L, pig FFA2-like receptor; pGAPDH, pig glyceraldehyde phosphate dehydrogenase; RT, reverse transcription.
All primers were synthesized by Invitrogen (China).
[a] The restriction sites added are underlined.

Osaka, Japan). The PCR products were cloned into pTA2 vector (Toyobo Co, Ltd) and sequenced. The cDNA sequence of each receptor gene was then determined by sequencing of at least 3 independent clones.

To examine whether pig *FFA2* (*pFFA2*) and *FFA2L* (*pFFA2L*) are functional in vitro, we subcloned the coding region of each receptor into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA, USA). The sequence of each receptor expression plasmid was further confirmed by sequencing before being used for functional studies.

2.5. Data mining, sequence alignment, and phylogenetic analysis

To determine whether both *FFA2* and *FFA2L* genes exist in other mammalian species, using *pFFA2* and *pFFA2L* cDNA sequences as references, we searched 42 sequenced mammalian genomes listed in Supplementary Table 1 (http://www.ensembl.org). The *FFA2* gene was identified in nearly all genomes except some poorly assembled genomes with many gaps (data not shown), whereas *FFA2L* predicted to encode a full-length receptor was only identified in 10 mammalian species, including the pig (Supplementary Table 1).

The deduced amino acid sequences of *pFFA2* and *pFFA2L* were aligned with those of other species by using the ClustalW program (BioEdit, Carlsbad, CA, USA). The putative transmembrane (TM) domains were predicted by using an online protein topology prediction tool TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Phylogenetic analysis was computed by using the program MEGA4 [20], in which the phylogenetic tree was constructed with the neighbor-joining method, and confidence was estimated with 1,000 bootstrap replicates, whereas sequences of FFA3 and FFA1 from several mammalian species were included as outgroups. The amino acid sequences of *FFA2* from non-mammalian vertebrates were not included in the alignment and phylogenetic analyses because of the complex duplication events of *FFA2* which occurred in these species.

2.6. Cell culture

Human embryonic kidney (HEK293) cells were maintained in Dulbecco modified Eagle medium (DMEM; low glucose) supplemented with 10% (vol/vol) fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Life Technologies, Inc, Grand Island, NY, USA) in a 90-cm culture dish (Nunc, Rochester, NY, USA). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 and were routinely subcultured every 3 d.

2.7. Functional characterization of pFFA2 and pFFA2L in cultured HEK293 cells

According to our previously established method [21,22], the functionalities of pFFA2 and pFFA2L were examined in HEK293 cells cotransfected with the receptor expression plasmid and a pGL4-SRE-luciferase reporter vector (vector name, pGL4.33[*luc2P*/SRE/Hygro] vector; Promega), which contains a serum responsive element (SRE) and drives the transcription of the luciferase reporter gene in response to MAPK/extracellular signal-regulated kinase activation. In brief, HEK293 cells were first subcultured on a 6-well plate and grown for 24 h before transfection. Then, the cell were cotransfected with a mixture that contained 700 ng of pGL4-SRE-luciferase reporter vector, 200 ng of receptor expression plasmid (or empty pcDNA3.1 vector), and 6 μL of Lipofectamine 2000 (Invitrogen). Transfected cells were incubated in fresh DMEM for an additional 24 h. Human embryonic kidney 293 cells were trypsinized and then cultured in a 96-well plate at 37°C for an additional 24 h before drug treatment. The SCFAs were diluted by serum-free DMEM to the indicated concentrations. After 6 h of treatment of SCFAs (serum-free medium used as a control), HEK293 cells were harvested with 1× passive lysis buffer for luciferase assay (Promega). The luciferase activity was measured by a Multimode Microplate Reader (TriStar LB 941; EG&G Berthold, Bad Wildbad, Germany) according to the manufacturer's instruction.

2.8. Statistical analysis

The luciferase activities in each treatment group were expressed as relative fold increase compared with the control group (without hormone treatment). The data were analyzed by nonlinear regression, followed by dose-response-stimulation by using GraphPad Prism version 5

software (GraphPad Software, San Diego, CA, USA). To validate the results, these experiments were repeated at least twice.

3. Results

3.1. Cloning the full-length cDNAs of pFFA2 and pFFA2L genes

To determine whether *FFA2* (also called *GPR43*) exists in the pig genome, using human *FFA2* cDNA sequence (NM_005306) as a template, we performed a search of the pig genome and identified the *FFA2* on chromosome 6, which shares high nucleotide identity (86%) to the human *FFA2* sequence. Strikingly, in addition to the identification of *FFA2* in the pig genome, a novel gene highly homologous to *FFA2* (85% nucleotide sequence identity) was also identified 21.7 kb away from *pFFA2* on the same chromosome (chromosome 6). According to its high amino acid sequence identity to pFFA2 and pharmacologic property examined in the next section, we designated this novel receptor as FFA2-like receptor with abbreviation as FFA2L in this study.

To obtain the full-length cDNA sequence of *pFFA2* and *pFFA2L*, using gene-specific primers, we amplified and cloned cDNA sequences that contained the complete ORF regions of the 2 receptors from pig intestine. The cloned *pFFA2* is 1059 bp in length and encodes a receptor of 329 amino acids (Accession no. JQ768799), which shares high amino acid sequence identity with that of humans (84%; NP_005297), cattle (81%; NP_001157256), mice (83%; NP_666299), and rats (84%; NP_001005877) (Fig. 1). Like FFA2 identified in other species, pFFA2 also contains a number of structural characteristics of class A GPCRs (rhodopsin family). These features include 7 TM domains (TM1–TM7), a Glu-Arg-Tyr (ERY) motif located at the bottom of TM3 critical for receptor conformation and function, and 2 cysteines (C81 and C163) in the first and second extracellular loops (ECL1 and ECL2) for the formation of an intramolecular disulfide bond [1]. Moreover, 2 arginines (R179, R254) and 1 histidine (H241) shown to be crucial for recognition of SCFAs are also fully conserved between pig and other species [8]. In addition, 3 putative *N*-glycosylation sites (NXS/T, X represents any amino acid residue except proline) were also identified in ECL2. Despite the high amino acid sequence identity of FFA2 noted among the species, 2 less-conserved regions were noted in ECL2 and the C-terminus.

The cloned *pFFA2L* is 1105 bp in length and encodes a receptor precursor of 329 amino acids (Accession no. JQ768798), which shares a relatively high degree of amino acid sequence identity with FFA2 of pigs (70%; JQ768799), humans (70%; NP_005297), cattle (63%; NP_001157256), mice (66%; NP_666299), and rats (68%; NP_001005877) and a comparatively lower identity (43%–45%) with FFA3, another receptor specific to SCFAs in mammals. Like pFFA2, pFFA2L also contains a number of conserved structural features, motifs, and amino acid residues. These include 7

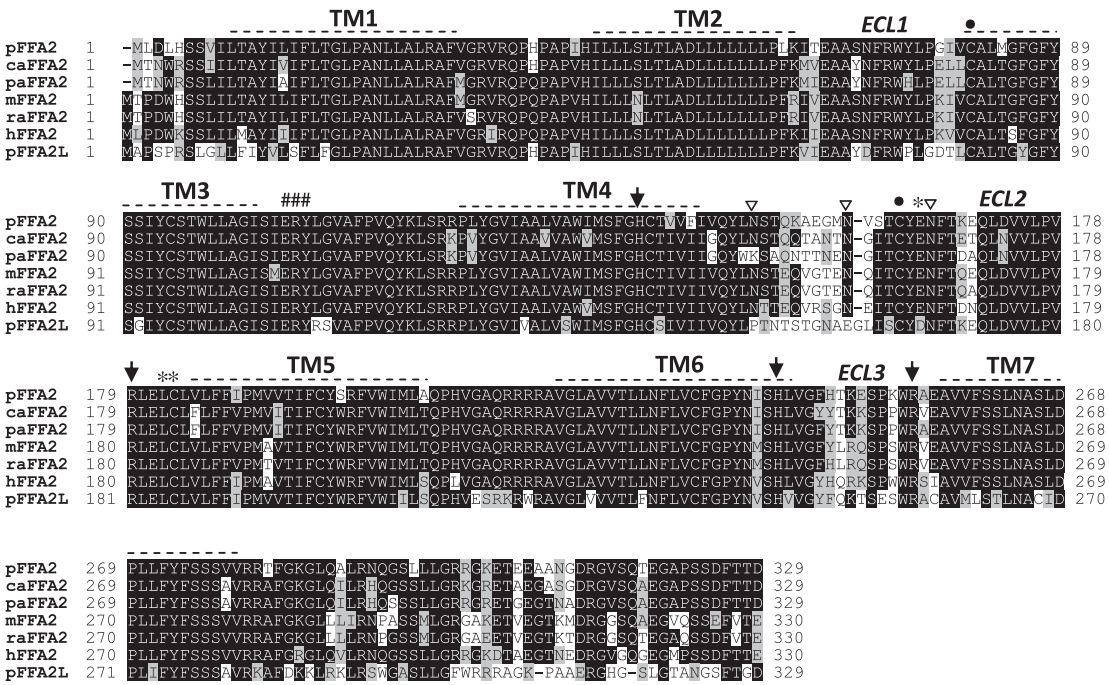

Fig. 1. Amino acid sequence alignment of pig FFA2 (pFFA2; JQ768799) with that of cat (caFFA2), giant panda (paFFA2), mouse (mFFA2; NP_666299), rat (rFFA2; NP_001005877), human (hFFA2; NP_005297), or with pig FFA2-like receptor (pFFA2L; JQ768798). Identical amino acids of FFA2 between pig and other species have a black background, and similar amino acids have a gray background. Horizontal dashed lines indicate the 7 putative transmembrane regions (TM1–TM7). The extracellular domains 1, 2, and 3 are also labeled as ECL1, ECL2, and ECL3, respectively. Arrows indicate the conserved basic amino acids (H139, R179, H241, and R254) known to be critical for recognition of SCFAs. Triangles indicate the locations of 3 putative *N*-glycosylation sites (NXT/S; where X represents any amino acid except proline); asterisks denote the amino acid residues shown to responsible for ligand selectivity of FFA2; # indicates the ERY motif fully conserved among species. FFA2, free fatty acid receptor 2; SCFA, short-chain fatty acid.

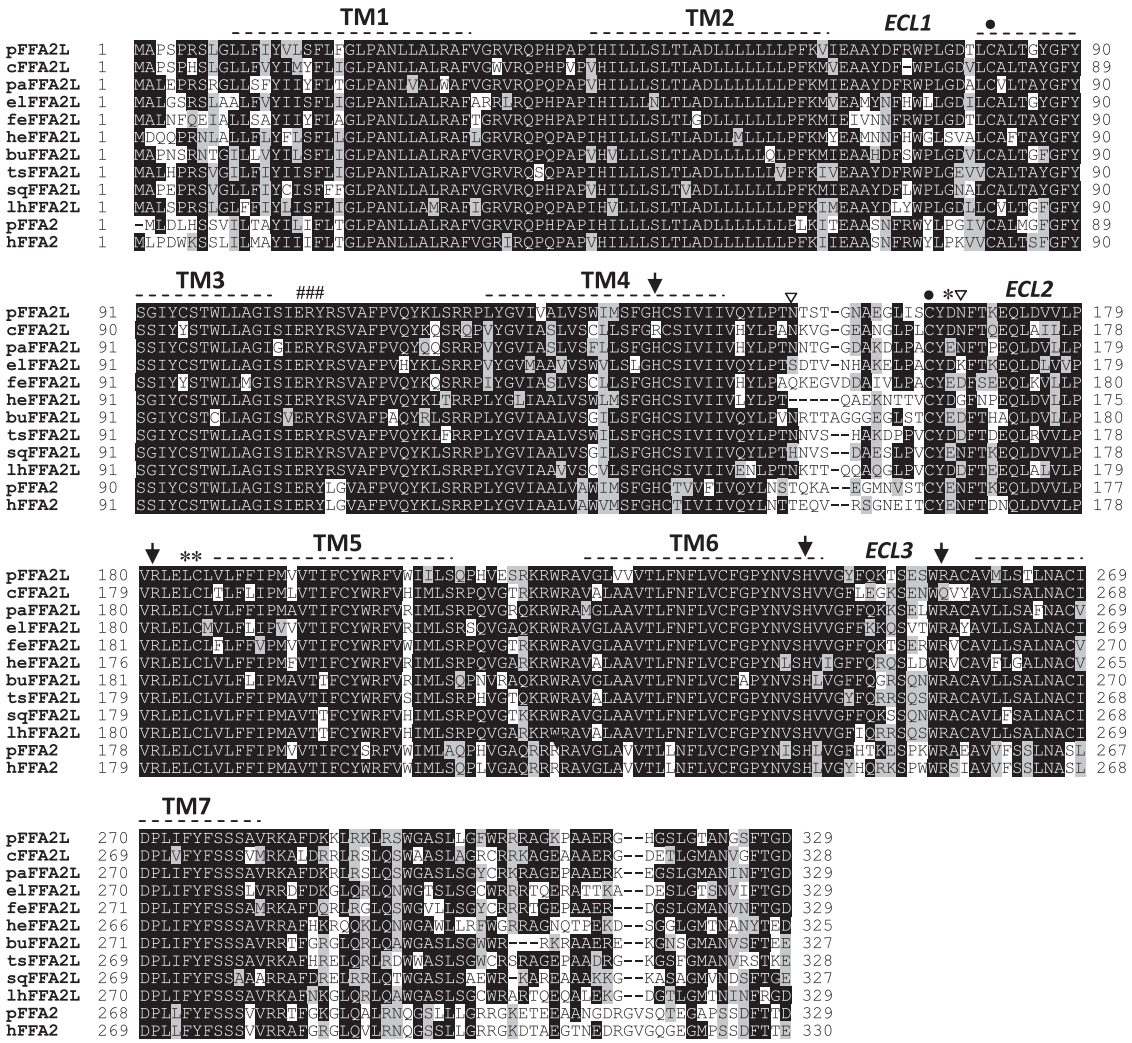

Fig. 2. Amino acid sequence alignment of pig FFA2L (pFFA2L; JQ768798) with that of cat (caFFA2L; XP_003998030), giant panda (paFFA2L; XP_002920936), African savanna elephant (elFFA2L; XP_003420849), ferret (feFFA2L), hedgehog (heFFA2L), bushbaby (buFFA2L), Chinese tree shrew (tsFFA2L; ELV09361), squirrel (sqFFA2L), lesser hedgehog tenrec (lhFFA2L), or with pig FFA2 (pFFA2; JQ768799) and human FFA2 (hFFA2: NP_005297). Identical amino acids between pig and other species have a black background, and similar amino acids have a gray background. Horizontal dashed lines indicate the 7 putative transmembrane regions (TM1–TM7). The extracellular domains 1, 2, and 3 are also labeled as ECL1, ECL2, and ECL3, respectively. Arrows indicate the conserved basic amino acids (H140, R181, H243, and R256) known to be critical for recognition of SCFAs. Asterisks denote the amino acid residues shown to responsible for ligand selectivity of FFA2; # represents the ERY motif fully conserved between species. The amino acid sequences of *FFA2L* are either predicted according to the genomic sequences of these species (http://www.ensembl.org) or retrieved from GenBank. FFA2L, FFA2-like receptor; SCFA, short-chain fatty acid.

TM domains (TM1–TM7), an ERY motif, 2 cysteines (C82 and C165) for disulfide bond formation, 3 basic residues at positions 181 (R181), 243 (H243), and 256 (R256) for recognition of SCFAs [8], and 2 putative *N*-glycosylation sites (NXS/T) in ECL2 (Fig. 2).

3.2. Identification of the novel FFA2L in 9 other mammalian species

To determine whether the novel *FFA2L* also exists in other mammalian species, using *pFFA2L* cDNA sequence as query (Accession no. JQ768798), we searched the sequenced genomes of 42 mammalian species, including humans (http://www.ensembl.org) (Supplementary Table 1), and successfully identified the *FFA2L* ortholog encoding a putative full-length receptor in 9 mammalian species, including cats (328 amino acids), giant pandas (329 amino acids), elephants (329 amino acids), ferrets (329 amino acids), hedgehogs (325 amino acids), bushbabies (327 amino acids), Chinese tree shrews (328 amino acids), squirrels (327 amino acids), and lesser hedgehog tenrec (329 amino acids). Expectedly, the predicted FFA2L identified from these species shares high amino acid sequence identity to pFFA2L (74%–80%) and mammalian FFA2 (63%–72%) but a comparatively lower identity to mammalian FFA3 (44%–48%). Moreover, 7 TM domains, the ERY motif, and 2 cysteines for disulfide bond formation were also highly conserved among species. Most importantly, the 3 polar residues

(corresponding to R181, H243, and R256 in pFFA2L) critical for SCFA recognition were fully conserved in nearly all species (Fig. 2), except 1 amino acid substitution (R256→Q256) noted in cat FFA2L.

Similar to the synteny of *FFA2* and *FFA2L* in pigs, *FFA2L* identified in all these species was also found to be located adjacent to *FFA2* on the same chromosome (Fig. 3), strongly suggesting that *FFA2L* is most likely originated from a tandem duplication of *FFA2* in the common ancestor of these species.

3.3. Functional characterization of pFFA2 and pFFA2L in cultured HEK293 cells

To determine whether both pFFA2 and pFFA2L can function as the 2 receptors specific to SCFAs and are capable of transducing signals, pFFA2 and pFFA2L were transiently expressed in HEK293 cells and subjected to treatment by 3 SCFAs, acetate (C2), propionate (C3), and butyrate (C4), and a long-chain fatty acid, DHA. Because activation of human FFA2 can simulate the intracellular MAPK/extracellular signal-regulated kinase signaling pathway [6,8], therefore, in this study, activation of pFFA2/pFFA2L on SCFA binding was monitored by a cell-based pGL4-SRE-luciferase reporter system, which could detect the receptor-mediated MAPK activation. As shown in Figure 4, all SCFAs could stimulate luciferase activity via activation of pFFA2 in a dose-dependent manner. The potencies of 3 SCFAs in activating pFFA2 are much similar (median effective concentration [EC_{50}] of acetate, 0.16 m*M*; EC_{50} of propionate, 0.62 m*M*; EC_{50} of butyrate, 0.29 m*M*). In contrast, pFFA2 expressed in HEK293 cells could not be activated by DHA (10^{-9} to 10^{-4} *M*) or by other long-chain fatty acids, including α-linolenic acid and linolenic acid (10^{-9} to 10^{-4} *M*) (data not shown). These findings clearly indicated that pFFA2 can function as a receptor specific to SCFAs. Like pFFA2, pFFA2L was also activated by acetate and propionate with high potencies (EC_{50} of acetate, 0.69 m*M*; EC_{50} of propionate, 5.3 m*M*) but not by butyrate (C4). Similarly, pFFA2L was not activated by DHA or by α-linolenic acid and linolenic acid (data not shown). These findings indicated that pFFA2L is also a functional receptor specific to SCFAs with a pharmacologic property similar, but not identical, to that of pFFA2. As an internal control, SCFAs failed to stimulate the luciferase activities of HEK293 cells transfected with an empty pcDNA3.1 vector, further confirming the specific actions of each SCFA on receptor activation (Fig. 4).

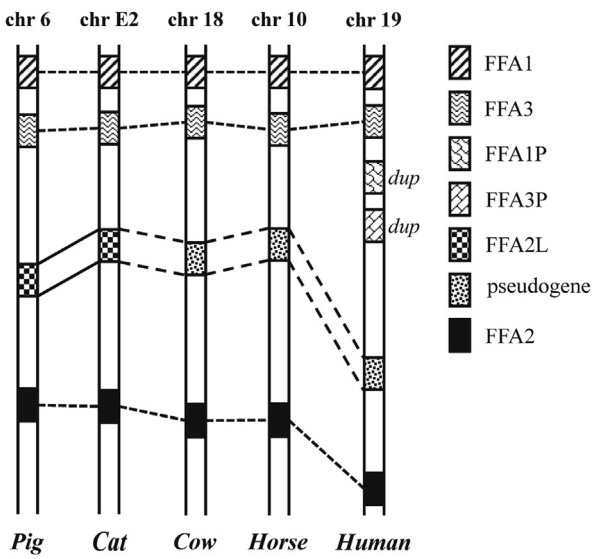

Fig. 3. Chromosomal localization of *FFA2* and *FFA2L* genes in pigs, cats, cows, horses, and humans. The *FFA2* and *FFA2L* genes are located on the same synteny conserved between pig and cat (or other species). *FFA2L* is likely generated by a tandem duplication of *FFA2* in mammalian lineage. Although *FFA2L* could not be identified in other species, such as cows, horses, and humans, the DNA fragment predicted to encode a truncated FFA2L could still be identified in these species, indicating that the duplicated *FFA2L* may have undergone pseudogenization (pseudo-FFA2L) and thus loss its biological function during speciation. Interestingly, an additional duplication event of *FFA1* (free fatty acid receptor 1, also called *GPR40*) and *FFA3* (also called *GPR41*) occurred in human genome may also lead to the generation of 2 pseudogenes: *FFA1P* (pseudo-GPR40) and *FFA3P* (pseudo-GPR42) [36]. chr, chromosome; FFA1, free fatty acid receptor 1; FFA2L, FFA2-like receptor; FFA3, free fatty acid receptor 3.

3.4. Tissue expression of FFA2 and FFA2L in adult pig tissues

To investigate the potential roles of *FFA2* and *FFA2L* in pigs, using RT-PCR, we examined the mRNA expression of both *FFA2* and *FFA2L* in 21 adult pig tissues, including subcutaneous adipose tissue and various parts of the GI tract. As shown in Figure 5, the PCR signal of *pFFA2* was noted in nearly all tissues examined except cerebrum, cerebellum, and hypothalamus, indicating its wide tissue expression. In contrast, the strong PCR signal of *pFFA2L* was only noted in the GI tract, including duodenum, ileum, cecum, proximal colon, distal colon, and rectum, and an extremely faint PCR signal was observed in liver and spleen. No signal of *pFFA2L* was detected in other pig tissues. In RT-negative control groups, no PCR signal was detected in all tissues examined (Fig. 5).

4. Discussion

In this study, *FFA2* (*GPR43*) and the novel *FFA2L* were cloned in pigs. Functional studies found that both pFFA2 and pFFA2L can act as the receptors specific to SCFAs with similar, but not identical, pharmacologic properties. Reverse transcription PCR assay found that *pFFA2* mRNA was widely expressed in most of the tissues examined, including the GI tract, whereas *pFFA2L* mRNA is nearly exclusively expressed in the GI tract. As in pigs, *FFA2L* has also been identified in several mammalian species, including elephants, bushbabies, cats, and pandas. To our knowledge, our findings represent the first to report that a novel *FFA2L* (GPR43-like receptor) exists in some mammalian species and functions in pigs.

The cloned pFFA2 has a length (329 amino acids) nearly identical to FFA2 identified in other mammalian species, and its deduced amino acid sequence also shares a remarkable degree of amino acid sequence identity to FFA2 reported in humans, rats, and mice. Moreover, a number of key structural features and amino acid residues, such as 7 TM domains, the ERY motif, and the key amino acid residues critical for recognition of SCFAs, were also highly conserved between pig and other species [1]. These

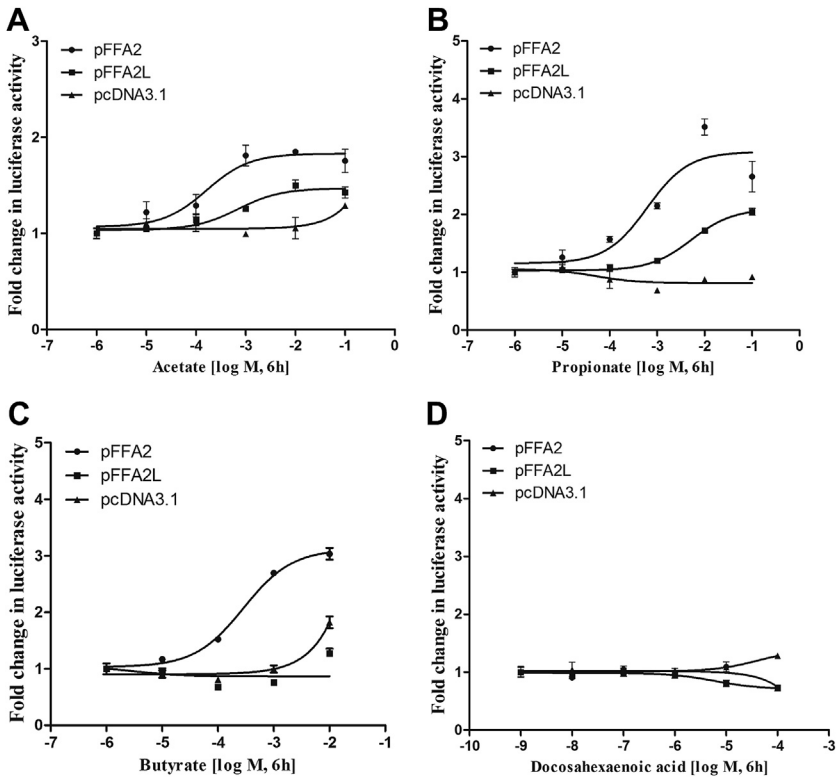

Fig. 4. Induction of the luciferase activities of HEK293 cells expressing pig FFA2 (pFFA2) or FFA2L (pFFA2L) in responses to graded concentrations of SCFAs (or DHA) treatments. HEK293 cells were transiently cotransfected with pFFA2 or pFFA2L expression plasmid and pGL4-SRE luciferase reporter construct, and were subjected to the ligand treatments for 6 h before luciferase activity measurement. (A) Acetate (10^{-6} *M* to 10^{-1} *M*; 6 h), (B) propionate (10^{-6} *M* to 10^{-1} *M*; 6 h); (C) butyrate (10^{-6} *M* to 10^{-2} *M*; 6 h), (D) DHA (10^{-9} *M* to 10^{-4} *M*; 6 h) treatments are shown. The cotransfection of empty pcDNA3.1 vector and pGL4-SRE-luciferase reporter construct into HEK293 cells was used as an internal control. Each data point represents the mean ± SEM of 3 replicates. DHA, docosahexaenoic acid; FFA2, free fatty acid receptor 2; FFA2L, FFA2-like receptor; HEK, human embryonic kidney; SCFA, short-chain fatty acid.

findings support that *pFFA2* is orthologous to *FFA2* of other mammalian species.

Strikingly, in addition to *FFA2* identified in pigs, a novel gene highly homologous to *pFFA2* was also identified. Like mammalian *FFA2*, *pFFA2L* coding region was also encoded by a single exon. Moreover, pFFA2L shares remarkable degree of amino acid sequence identity (70%) to pFFA2, including the amino acid residues critical for SCFA recognition fully conserved between the 2 receptors [8]. The cloning of *pFFA2L* from the intestine further confirmed the existence and expression of *pFFA2L* in pigs.

Similar to the finding in pigs, *FFA2L* gene encoding a full-length receptor, which is located on the neighboring region of *FFA2*, has also been identified in the genomes of 9 mammalian species among 42 species examined. Amino acid sequence alignment also showed that all the predicted full-length FFA2L shares a high degree of amino acid sequence identity to FFA2 (63%–72%), and comparatively low identities either to FFA3 (44%–48%) or to FFA1 (25%), a receptor structurally related to FFA2 for medium- and long-chain free fatty acids [23]. Despite failure to identify the full-length FFA2L in the other remaining 32 mammalian genomes, including the human genome, the DNA sequence, which is predicted to encode the partial or truncated FFA2L, has been identified in most mammalian species, including human (Fig. 3). The failure in identifying a complete ORF (or partial homologous sequence) of *FFA2L* in these species is likely because of pseudogenization, or elimination, of the duplicated gene during speciation. However, the presence of *FFA2L* or pseudo-*FFA2L* gene sequences in most mammalian genomes examined (Supplementary Table 1), together with the high degree of sequence similarity shared between *FFA2* and *FFA2L*, strongly suggest that *FFA2L* is generated by a tandem duplication event of *FFA2L* that occurred in the last common ancestor of these species. This idea was further supported by the phylogenetic analysis. *FFA2* and *FFA2L* genes of all mammalian species were grouped into the same cluster with a high bootstrap value (100), whereas *FFA3* and *FFA1* from all mammalian species form 2 separate clades, indicating the much closer evolutionary origin between *FFA2* and *FFA2L* genes (Fig. 6). Interestingly, the phylogentic analysis also showed that FFA2 from the 3 most primitive mammalian species, platypus, opossum, and Tasmanian devil, seems to diverge much earlier from *FFA2* or *FFA2L* of Eutherian mammalian species. This finding, together with the failure to identify an extra copy of *FFA2* in the genomes of opossum and Tasmanian devil, also points to a possibility that *FFA2* duplication was likely to have occurred in the Eutherian lineage, although this hypothesis needs further verification.

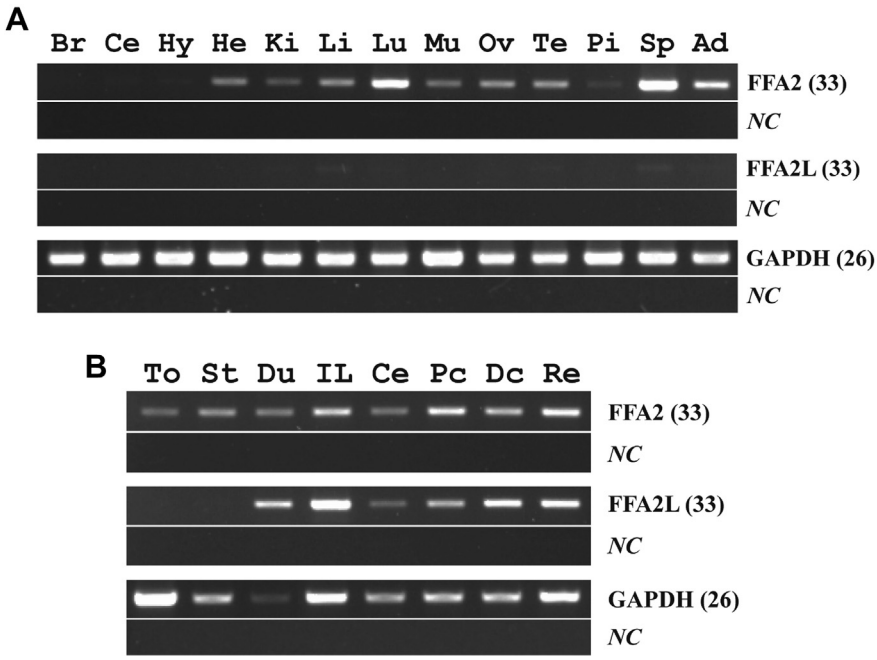

Fig. 5. Reverse transcription PCR detection of the mRNA expression of *FFA2* and *FFA2L* in adult pig tissues (A): cerebrum (Br), cerebellum (Ce), hypothalamus (Hy), heart (He), kidney (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), testis (Te), pituitary (Pi), spleen (Sp), subcutaneous adipose tissue (Ad); and various parts of the gastrointestinal tract (B), including tongue (To), stomach (St), duodenum (Du), ileum (IL), cecum (Ce), proximal colon (Pc), distal colon (Dc), and rectum (Re). Number in bracket indicates PCR cycle number used. No PCR band of expected sizes was amplified from RT negative controls (NC) of all tissues examined. FFA2, free fatty acid receptor 2, FFA2L, FFA2-like receptor; GAPDH, glyceraldehyde phosphate dehydrogenase.

The remarkable degree of amino acid sequence identity, particularly those fully conserved residues critical for SCFA recognition between FFA2 and FFA2L [2], suggests that both receptors may function as the receptors specific for SCFAs in pigs. With the use of a pGL4-SRE-luciferase reporter assay, we demonstrated that both receptors were activated by SCFAs but not by long-chain fatty acids, suggesting that both FFA2 and FFA2L can function as the receptors specific to SCFAs and are capable of activating MAPK signaling pathway as previously reported [6,8]. However, pFFA2 and pFFA2L have a similar, but not identical, pharmacologic property. Like FFA2 characterized in humans and mice, pFFA2 could be activated by acetate, propionate, and butyrate with potencies within micromolar range [5–7]. In contrast, pFFA2L could be activated only by acetate and propionate, but not by butyrate, suggesting the ligand selectivity of pFFA2L. It is reported that ligand selectivity of human FFA2 was determined by 3 amino acids (E166, L183, C184) lining the FFA2 binding sites. Replacement of the 3 residues of human FFA2 with those in human FFA3 residues could change the architecture of binding pocket and facilitate FFA3 ligand binding [24]. In pFFA2L, we also noted that E166 was substituted by D166 (E166→D166), whereas L183 and C184 were still conserved, suggesting that this substitution may be associated with the ligand selectivity of pFFA2L. In addition to the aforementioned 3 residues, other residues such as H140 were also suggested to regulate the receptor function and ligand selectivity [8]. Moreover, pFFA2L and pFFA2 also display notable differences in their ECL2, an important region known to be involved in ligand binding, selectivity and entry of class-A GPCRs [25]. Therefore, how these amino acids or region(s) could coordinate their actions in ligand recognition and confer the ligand selectivity of FFA2L requires further investigation.

The existence of 2 FFA2s (FFA2 and FFA2L) in pigs and 9 other mammalian species, together with the fact that FFA3 is also a functional receptor for SCFAs in mammals, including pigs (our unpublished data) [26], strongly suggested that the biological actions of SCFAs are likely mediated by 3 receptors, FFA2, FFA2L, and FFA3, in these mammalian species. However, it should be noted that the substitutions of key residues at the orthosteric binding pocket known to be critical for endogenous agonist recognition were noted in cat FFA2L, such as R255→Q255, whose mutation could completely abolish the responses of human FFA2 and FFA3 to SCFAs [8]. Therefore, further experimental studies are needed to confirm whether both FFA2 and FFA2L are functional receptors specific to SCFAs in these species as found in pigs, or *FFA2L* in some species only represents a pseudogene with a complete ORF, as previously reported in human *GPR42* gene [27]. Clearly, these studies will help to uncover the underlying mechanisms of SCFA actions in these mammalian species.

Although both FFA2 and FFA2L have much closer evolutionary origin and similar pharmacologic property, they have nonidentical spatial expression pattern. Free fatty acid receptor 2 mRNA was detected to be widely expressed in nearly all pig tissues, including various parts of the GI tract, whereas *FFA2L* mRNA expression was mainly restricted in the GI tract. Our finding is also partially

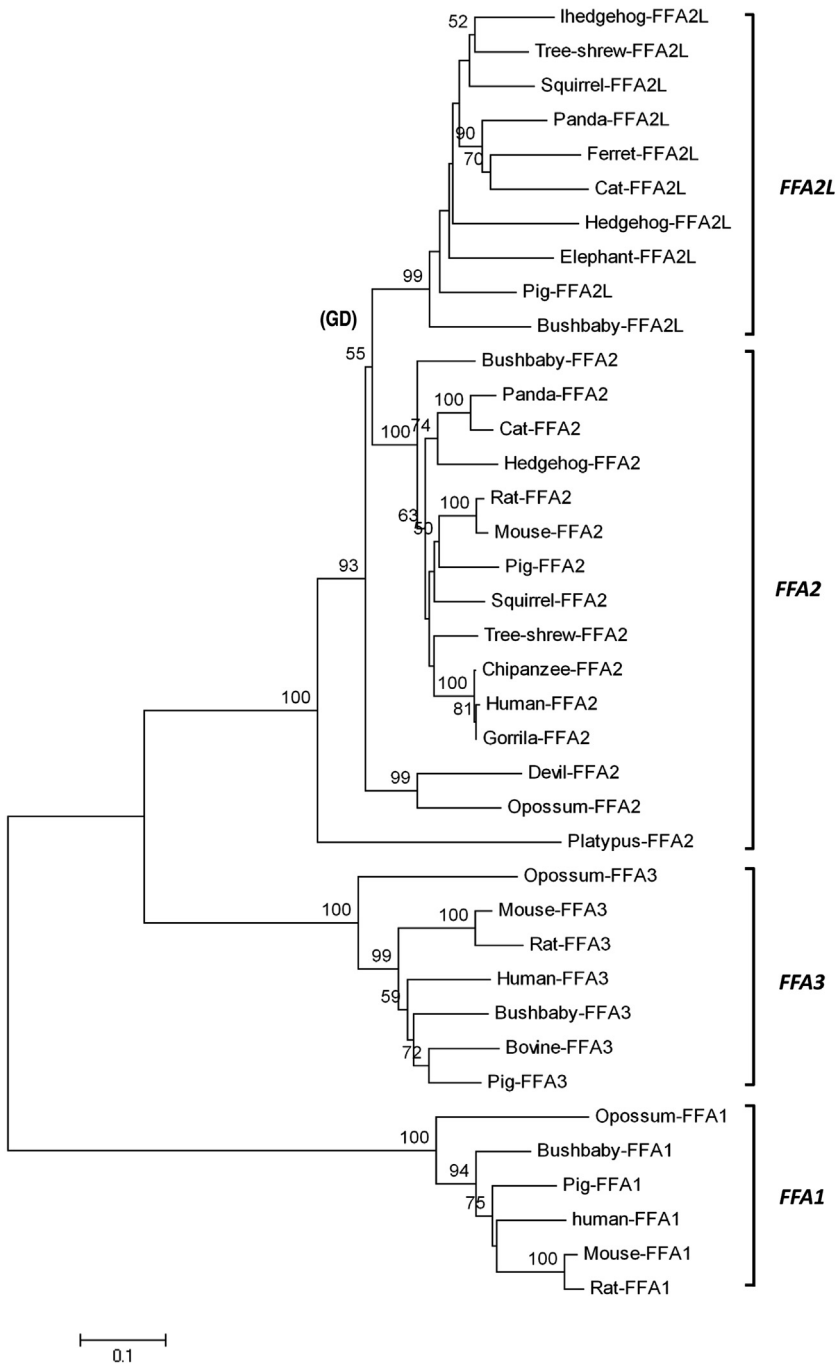

Fig. 6. Phylogenetic tree shows the evolutionary relationship of pig *FFA2*, *FFA2L*, *FFA3*, and *FFA1* with the receptor genes from other species, including lesser hedgehog tenrec (Ihedgehog), Chinese tree shrew, squirrel, giant panda, ferret, cat, hedgehog, elephant, bushbaby, rat, mouse, chimpanzee, human, gorilla, Tasmanian devil, opossum, and platypus. The tree was based on the full amino acid sequences of *FFA*s and was constructed by the neighbor-joining method with bootstrap analysis (1000 replicates) in MEGA 4. Numbers at the nodes indicate bootstrap values in percentage (bootstrap values <50 are not shown). Bootstrap values (>50) are shown at branch points. *FFA2* and *FFA2L* identified in pigs and other mammalian species were likely generated by a tandem GD event that occurred in the last common ancestor of these species. The sequences used were either retrieved from GenBank or predicted according to the genomic sequences of these species (http://www.ensembl.org). FFA1 (GPR40), free fatty acid receptor 1; FFA2 (GPR43), free fatty acid receptor 2; FFA2L (GPR43-like receptor), FFA2-like receptor; FFA3 (GPR41), free fatty acid receptor 3; GD, gene duplication.

coincided with a recent study in which *FFA2* mRNA was found to be expressed in the small intestine, cecum, and colon of 2-wk and adult pigs [17]. Similar to the findings in pigs, *FFA2* has also been reported to be expressed in ileum and colon of humans, rats, and mice [12,14,28], where activation of *FFA2* by SCFA binding is involved in the

regulation of multiple GI tract functions, including stimulation of glucagon-like peptide 1 and peptide YY secretion from enteroendocrine L cells and 5-hydroxytryptamine release from mast cells [13,15], control of the GI motility [29], regulation of colonic inflammation [4,30], and suppression of colon carcinogenesis [16]. The overlapping mRNA expression of *FFA2* and *FFA2L* in the pig GI tract, together with the presence of high concentrations of acetate, propionate, and butyrate in intestinal luminal contents of growing pigs under feeding conditions [17], also strongly suggests that in pig intestine FFA2, FFA2L, and FFA3 may mediate the biological actions of SCFAs, the major metabolites produced by gut microbiota (Figs. 4 and 5) [17]. The situations in pigs also differs from that in humans, rats, and mice, in which only FFA2 and FFA3 are involved in mediating the actions of SCFAs in the GI tract. In addition to the mRNA expression of *FFA2* and *FFA2L* detected in the lower part of the GI tract, the mRNA expression of *FFA2*, but not *FFA2L*, was detected to be expressed in stomach and tongue. Our finding is also consistent with the finding in rats, in which *FFA2* mRNA expression was detected in esophagus and stomach, although its physiological relevance in pigs and other species awaits further investigation [31].

Evidence is growing that *FFA2* is expressed in adipose tissue and involved in the regulation of adipogenesis and inhibition of lipolytic activity both in vivo and in vitro [9–11]. Recently, Kimura et al [32] showed that mice with FFA2 (GPR43) overexpressed specifically in adipose tissue were lean even when fed with a high-fat diet, and *FFA2*-deficient mice were obese with increased fat mass on a normal diet. These findings suggested that adipose tissue is also a main target site of SCFAs produced by gut microbiota, and SCFAs are likely to be the novel signaling molecules involved in the control of body energy stores. In this study, we also found that only FFA2, but not FFA2L, could be easily detected in adipose tissue of pigs, supporting that adipose tissue is also a target site for SCFA actions in pigs [33], as reported in other mammalian species, including cattle [18]. The expression of *FFA2* in pig adipose tissue also makes pigs an ideal model to decipher how SCFAs produced by gut microbiota could regulate adipogenesis and fat mass accumulation, which is also a phenotypic trait of great economic importance for farm animals.

In addition to adipose tissue and GI tract, *FFA2* mRNA was also detected to be expressed in a number of tissues, including spleen, heart, kidney, liver, lung, muscle, ovary, testis, and pituitary. Our finding is also partially consistent with the finding in cattle, in which *FFA2* expression was observed in nearly all tissues examined [34]. Considering the relatively high concentrations of SCFAs (eg, acetate) present in animal serum [35], the wide tissue expression of *FFA2* in pigs and cattle also strongly implies a much wider range of roles of SCFAs played in pigs and other mammalian species than previously proposed [2].

In summary, we cloned the *FFA2* and the novel *FFA2L* from pig intestine. Functional study found that both FFA2 and FFA2L can function as the 2 receptors specific to SCFAs with the similar, but not identical, pharmacologic property. Moreover, the novel *FFA2L* was identified in 9 other mammalian species. Reverse transcription PCR assay found that both *FFA2* and *FFA2L* are coexpressed in the GI tract and that *FFA2* displays a much wider tissue distribution than *FFA2L* in pigs. Evidence presented here, for the first time, raised a novel concept that the 3 structurally related receptors, FFA2, FFA2L, and FFA3, may mediate the biological actions of SCFAs produced by gut microbes in some mammalian species, such as pigs.

Acknowledgments

This work was supported by grants from the Ministry of Agriculture of China (2009zx08009-145B) and the National Natural Science Foundation of China (31272436, J1103518).

References

[1] Stoddart LA, Smith NJ, Milligan G. International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharmacol Rev 2008;60:405–17.
[2] Bindels LB, Dewulf EM, Delzenne NM. GPR43/FFA2: physiopathological relevance and therapeutic prospects. Trends Pharmacol Sci 2013;34:226–32.
[3] Delzenne NM, Neyrinck AM, Backhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 2011;7:639–46.
[4] Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 2009;461:1282–6.
[5] Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, et al. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 2003;278:11312–9.
[6] Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 2003;278:25481–9.
[7] Nilsson NE, Kotarsky K, Owman C, Olde B. Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 2003;303:1047–52.
[8] Stoddart LA, Smith NJ, Jenkins L, Brown AJ, Milligan G. Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. J Biol Chem 2008;283:32913–24.
[9] Hong YH, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, et al. Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. Endocrinology 2005;146:5092–9.
[10] Ge H, Li X, Weiszmann J, Wang P, Baribault H, Chen JL, et al. Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology 2008;149:4519–26.
[11] Dewulf EM, Cani PD, Neyrinck AM, Possemiers S, Van Holle A, Muccioli GG, et al. Inulin-type fructans with prebiotic properties counteract GPR43 overexpression and PPARgamma-related adipogenesis in the white adipose tissue of high-fat diet-fed mice. J Nutr Biochem 2011;22:712–22.
[12] Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, et al. Short-chain fatty acid receptor, GPR43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 2006;324:353–60.
[13] Freeland KR, Wolever TM. Acute effects of intravenous and rectal acetate on glucagon-like peptide-1, peptide YY, ghrelin, adiponectin and tumour necrosis factor-alpha. Br J Nutr 2010;103:460–6.
[14] Kaji I, Karaki S, Tanaka R, Kuwahara A. Density distribution of free fatty acid receptor 2 (FFA2)-expressing and GLP-1-producing enteroendocrine L cells in human and rat lower intestine, and increased cell numbers after ingestion of fructo-oligosaccharide. J Mol Histol 2011;42:27–38.
[15] Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, et al. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 2012;61:364–71.

[16] Tang Y, Chen Y, Jiang H, Robbins GT, Nie D. G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. Int J Cancer 2011;128:847–56.
[17] Haenen D, Zhang J, Souza da Silva C, Bosch G, van der Meer IM, van Arkel J, et al. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. J Nutr 2013;143:274–83.
[18] Smith SB, Go GW, Johnson BJ, Chung KY, Choi SH, Sawyer JE, et al. Adipogenic gene expression and fatty acid composition in subcutaneous adipose tissue depots of Angus steers between 9 and 16 months of age. J Anim Sci 2012;90:2505–14.
[19] Wang Y, Wang CY, Wu Y, Huang G, Li J, Leung FC. Identification of the receptors for prolactin-releasing peptide (PrRP) and Carassius RFamide peptide (C-RFa) in chickens. Endocrinology 2012;153:1861–74.
[20] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.
[21] Ho JC, Jacobs T, Wang Y, Leung FC. Identification and characterization of the chicken galanin receptor GalR2 and a novel GalR2-like receptor (GalR2-L). Gen Comp Endocrinol 2012;179:305–12.
[22] Kwok AH, Wang Y, Leung FC. Molecular characterization of prostaglandin F receptor (FP) and E receptor subtype 3 (EP3) in chickens. Gen Comp Endocrinol 2012;179:88–98.
[23] Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, et al. The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 2003; 278:11303–11.
[24] Schmidt J, Smith NJ, Christiansen E, Tikhonova IG, Grundmann M, Hudson BD, et al. Selective orthosteric free fatty acid receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. J Biol Chem 2012;286:10628–40.
[25] Peeters MC, van Westen GJ, Li Q, AP IJ. Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. Trends Pharmacol Sci 2011;32:35–42.
[26] Ulven T. Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. Front Endocrinol (Lausanne) 2012;3:111.
[27] Brown AJ, Jupe S, Briscoe CP. A family of fatty acid binding receptors. DNA Cell Biol 2005;24:54–61.
[28] Karaki S, Tazoe H, Hayashi H, Kashiwabara H, Tooyama K, Suzuki Y, et al. Expression of the short-chain fatty acid receptor, GPR43, in the human colon. J Mol Histol 2008;39:135–42.
[29] Tazoe H, Otomo Y, Kaji I, Tanaka R, Karaki SI, Kuwahara A. Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. J Physiol Pharmacol 2008;59(suppl 2):251–62.
[30] Sina C, Gavrilova O, Forster M, Till A, Derer S, Hildebrand F, et al. G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol 2009; 183:7514–22.
[31] Dass NB, John AK, Bassil AK, Crumbley CW, Shehee WR, Maurio FP, et al. The relationship between the effects of short-chain fatty acids on intestinal motility in vitro and GPR43 receptor activation. Neurogastroenterol Motil 2007;19:66–74.
[32] Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, et al. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 2013;4: 1829.
[33] Hou Z, Sun C. [Transcriptional expression of GPR43 gene in adipose tissue and primary cultured adipocytes of pig]. Sheng Wu Gong Cheng Xue Bao 2008;24:1361–6 [in Chinese].
[34] Wang A, Gu Z, Heid B, Akers RM, Jiang H. Identification and characterization of the bovine G protein-coupled receptor GPR41 and GPR43 genes. J Dairy Sci 2009;92:2696–705.
[35] Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 1987;28:1221–7.
[36] Liaw CW, Connolly DT. Sequence polymorphisms provide a common consensus sequence for GPR41 and GPR42. DNA Cell Biol 2009; 28:555–60.

Supplementary Table 1
FFA2L gene (or pseudo-*FFA2L* sequences) identified in mammalian species.

Species name	Chromosome/scaffold	FFA2L (length, aa)	Fragment/pseudo-FFA2L
Pig *Sus scrofa*	Chromosome 6	329	
Cat *Felis catus*	Chromosome E2	328	
Elephant *Loxodonta Africana*	Scaffold_99	329	
Ferret *Mustela putorius furo*	Scaffold: GL897008.1	329	
Hedgehog *Erinaceus europaeus*	Scaffold_344550:	325	
Lesser hedgehog tenrec *Echinops telfairi*	Scaffold_241584	329	
Panda *Ailuropoda melanoleuca*	Scaffold: GL192794.1	329	
Chinese tree shrew *Tupaia chinensis*	ELV09361[a]	328	
Squirrel *Ictidomys tridecemlineatus*	Scaffold: JH393380.1	327	
Bushbaby *Otolemur garnettii*	Scaffold: GL873619	327	
Alpaca *Vicugna pacos*	Scaffold_55430		Pseudogene
Armadillo *Dasypus novemcinctus*	Scaffold_19124		Pseudogene
Baboon *Papio hamadryas*	Scaffold: Contig722597_Contig439379		NI
Chimpanzee *Pan troglodytes*	Chromosome 19		Pseudogene
Chinese hamster, *Cricetulus griseus*	Scaffold: JH000588		NI
Cow *Bos taurus*	Chromosome18		Pseudogene
Dog *Canis lupus familiaris*	Chromosome 1		Pseudogene
Dolphin *Tursiops truncates*	Scaffold_102216:		NI
Gibbon *Nomascus leucogenys*,	Scaffold: GL397480.1		Pseudogene
Gorilla *Gorilla gorilla gorilla*	Chromosome: 19		NI
Guinea pig *Cavia porcellus*	Scaffold_56		Pseudogene
Horse *Equus caballus*	Chromosome 10		Pseudogene
Human *Homo sapiens*	Chromosome: 19		Pseudogene
Macaque *Macaca mulatta*	Chromosome 19		Pseudogene
Marmoset *Callithrix jacchus*	Chromosome: 22		NI
Megabat *Pteropus vampyrus*	Scaffold 7501		Pseudogene
Microbat *Myotis lucifugus*	Scaffold: GL430011		Pseudogene
Mouse lemur *Microcebus murinus*	Scaffold_1634		NI
Mouse *Mus musculus*	Chromosome: 7		NI
Olive baboon *Papio anubis*,	Chromosome: 19		Pseudogene
Opossum *Monodelphis domestica*	Chromosome 4		NE
Pika *Ochotona princes*	Scaffold_26467		pseudogene
Orangutan *Pongo abelii*	Chromosome: 19		NI
Rabbit *Oryctolagus cuniculus*	Scaffold GL018786		Pseudogene
Platypus *Ornithorhynchus anatinus*	Contig21033		NE
Rat *Rattus norvegicus*	Chromosome: 1		NI
Sheep *Ovis aries*	Chromosome: 14		Pseudogene
Shrew *Sorex araneus*	Scaffold: JH798147.1		Pseudogene
Sloth *Choloepus hoffmanni*	Scaffold_119259		Pseudogene
Squirrel monkey *Saimiri boliviensis*	Scaffold: JH378197.1		NI
Tarsier *Tarsius syrichta*	Scaffold_10264		Gaps
Tasmanian devil *Sarcophilus harrisii*	Scaffold: GL849758.1		NE

Abbreviations: FFA2L, free fatty acid receptor 2-like receptor; NE, *FFA2L* may not exist in the genomes of these species; NI, *FFA2L*/*pseudo-FFA2L* sequences were not identified in these species probably because of either gaps in their genomes or gene lost in these species.
[a] This sequence was retrieved from GenBank.