Identification of the Porcine G Protein-Coupled Receptor 41 and 43 Genes and Their Expression Pattern in Different Tissues and Development Stages

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Abstract

Short-chain fatty acids (SCFAs) are not only an important energy source, but they also play a regulatory role in various physiological processes in humans and rodents. Current studies, mostly in humans and rodents, have revealed that SCFAs act as endogenous ligands for G protein-coupled receptor GPR41 and GPR43. Whether proteins similar to human GPR41 and GPR43 mediate the regulatory effects of SCFAs in swine remains unclear to date. The aims of this study were to determine whether GPR41 and GPR43 genes are expressed in porcine different tissues; and whether the expression of GPR41 and GPR43 is tissue-specific and/or time-associated. The alignment results showed that pig chromosome 6 contained GPR41 and GPR43 genes. Reverse transcription polymerase chain reaction (RT-PCR) indicated that GPR41 and GPR43 were expressed in porcine various tissues. The 2218 bp and 1908 bp nucleotide sequence representing the full-length cDNA sequence of porcine GPR41 and GPR43 was obtained from the ileum and spleen using rapid amplification of cDNA ends (RACE), which were capable of encoding 335 and 329 amino acid sequences, respectively. The structure prediction revealed that porcine GPR41 and GPR43 proteins had seven putative trans-membrane domains. The real-time PCR results indicated that GPR41 and GPR43 were expressed throughout the developmental stages in a tissue-specific and time-associated manner. GPR41 and GPR43 were most highly expressed in the ileum (P<0.01) and the spleen (P<0.01), respectively. Western blot results showed that porcine GPR41 and GPR43 proteins were expressed in a variety of porcine tissues, including the spleen, ileum, colon, and adipose tissue. In situ GPR41 and GPR43 immunoreactivities were observed through immunohistochemistry in the spleen, ileum, colon, and adipose tissue. In conclusion, the pig genome encoded GPR41 and GPR43 genes, and these two genes were detected in a variety of porcine tissues and expressed in tissue-specific and time-associated manner.

Introduction

Short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, can supply 15~24% of net energy for maintenance in growing and finishing pigs [1,2]. In addition to providing energy, SCFAs play a regulatory role in various physiological processes. Propionate is capable of inhibiting hepatic cholesterol synthesis in humans [3], reducing food intake [4], and improving tissue insulin sensitivity [5]. Propionate, along with acetate, may be involved in the regulation of adipogenesis [6] and increased cycling leptin level [7]. Butyrate enhances the differentiation and proliferation of colonic mucosa cells [8], ameliorates mucosal inflammation [9], and modulates visceral sensitivity [10].

The precise mechanisms underlying the above regulatory effects of SCFAs are poorly understood. Recent studies employing the “reverse pharmacology” approach have reported that SCFAs acted as endogenous ligands for orphan G protein-coupled receptor 41 and GPR43 [11-13]. It has also been reported that GPR41 mediated the stimulatory effect of SCFAs on leptin production in adipocytes [7] and the effect of gut microbiota on host adiposity and energy balance in mice [14], and that GPR43 mediated the effect of SCFAs on the promotion of adipogenesis [6] and inhibition of lipolysis in vitro [15]. Dewulf et al. [16] demonstrated that inulin-type fructans (ITFs), which can be fermented by gut microbiota for SCFA production, counteracted PPARγ overexpression and PPARγ activation induced by a high-fat diet in the adipose tissue of mice. Bjursell et al. [17] found that GPR43 knockout protected mice from obesity and dyslipidemia induced by a high-fat diet. These rodent’s original results indicated that GPR41 and GPR43 might be the underlying mechanism of SCFA-associated physiological processes.

To date, the majority of research studies on GPR41 and GPR43 have been on humans and rodents. The identification of porcine GPR41 and GPR43 and their functions in physiological processes remains to be elucidated. In this study, we tried to determine whether and where GPR41 and GPR43 genes are expressed in swine, and the expression pattern of these two genes in different tissues and developmental stages.
Materials and Methods

All surgical and animal care procedures in this study followed protocols approved by Experimental Animal Care and Use guidelines (Chinese Science and Technology Committee, 1988).

Tissue collection and RNA extraction

Various porcine tissues (liver, spleen, ileum, colon, heart, kidney, adipose tissue, and skeletal muscle) were collected from each three Landrace×Yorkshire pigs slaughtered at one (newborn), 25 (weaning), 35 (nursing), 70 (nursing), 115 (growing), and 160 (finishing) days, and stored at −80°C until total RNA and membrane protein extraction.

Reverse transcription polymerase chain reaction (RT-PCR)

The specific primers of GPR41, GPR43, and GAPDH genes (reference gene) were designed using Premier Primer 5 based on the sequences of predicted porcine GPR41 (Accession number: XM_003127053.2) and GPR41 (Accession number: XM_003127046.3), as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Accession number: NM_001206359.1). All primers used in this study were synthesized by Invitrogen (Shanghai, China) and are presented in Table 1.

RT-PCR was used to detect the expression of porcine GPR41 and GPR43 in various tissues. 30 μg of total RNA was pooled equally from three 160-day-old pigs and digested with 10 U of DNase I (Takara, Dalian) at 37°C for 30 min, followed by phenol-chloroform-isooamyl alcohol (25:24:1) and chloroform-isooamyl alcohol (24:1) extraction. 2 μg of the DNase I-digested RNA were reverse-transcribed to cDNA in a total volume of 20 μL in the present or absence of PrimeScript RTase with oligo dT primer and random hexamers (Takara, Dalian). The conditions of these PCRs were 35 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 10 min. The amplified products were detected by 2% agarose gel to characterize the distribution of GPR41 and GPR43 in porcine tissues.

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE) was used to amplify the 3′ and 5′ end regions of porcine GPR41 and GPR43 mRNA, using SMART RACE cDNA Amplification (Clontech, Beijing).

The total ileum and spleen RNA from the three 160-day-old pigs was used for RACE of porcine GPR41 and GPR43, respectively. For 3′ RACE, the first-strand cDNA was transcribed from 2 μg total RNA using the primer of 3′ RACE P1. The cDNA were used as the templates in subsequent nested PCR to amplify the 5′ end of porcine GPR41 and GPR43 cDNA using parallel primer sets of 3′ RACE P2 with 3′ RACE 41-1 or 3′ RACE 43-1, and 3′ RACE P2 with 3′ RACE 41-2 or 3′ RACE 43-2 (Table 1). For 5′ RACE, 2 μg total RNA was pooled for the first-strand cDNA synthesis using the primer of oligo dT, and a TdT tail was added to the cDNA. The cDNA were used as the templates in subsequent nested PCR to amplify the 5′ end sequences of porcine GPR41 and GPR43 cDNA using parallel primer sets of 5′ RACE P2 with 5′ RACE 41-1 or 5′ RACE 43-1, and 5′ RACE P2 with 5′ RACE 41-2 or 5′ RACE 43-2 (Table 1).

Plasmid construction and real-time PCR

The mRNA expression profiles of GPR41 and GPR43 in the liver, spleen, ileum, colon, and adipose tissue with respect to the different development stages (1 d, 25 d, 35 d, 70 d, 115 d and 160 d) were determined with the real-time fluorescent quantitative PCR method, using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, NY).

The primers GPR41-Q and GPR43-Q were used to amplify porcine GPR41 and GPR43 genes from ileum and spleen cDNA, respectively. The amplified products were cloned to the pGEM-T Easy vector (Promega, Madison), which was subsequently transformed to TOP 10 competent cells (Tiangen, Shanghai). The plasmids with specific amplified products, as standard substances for absolute quantification of GPR41 and GPR43 mRNA, were extracted using a SunShineclean™ Plasmid Mini Extraction Kit (Sunshinebio, Nanjing). The calibration curves were performed using a series of diluted plasmid constructs. The slopes of the calibration curves were 3.53 and 3.25 for GPR41 and GPR43, respectively, indicating that the efficiency of qPCR was acceptable (data not shown). There was only one amplified product for each pair of primers shown in melting curves, indicating that the primers we used were specific (data not shown).

One μg total RNA of various tissues from individual pigs at each development stage was first used for reverse transcription (PrimeScript RT reagent Kit with gDNA Eraser; Takara, Dalian). 2 μL of cDNA was mixed with 2× SYBR Premix Ex Taq with Tli RNaseH Plus (Takara, Dalian) using the primers GPR41-Q and GPR43-Q in a total volume of 20 μL. The conditions of these PCRs were 40 cycles of 95°C for 3 s and 58.5°C for 31 s, followed by a dissociation curve of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. The qPCR data was analyzed with 7300 System SDS software v1.3.0 (Applied Biosystems, NY). The gene copies were calculated according to the calibration curves. The
target gene expression results are presented as copies per 1 microgram total RNA [10].

Western blot
About 100 mg of certain tissues were pooled equally from three 160-day-old pigs for membrane protein extraction using a Membrane and Cytoplasm Protein Extraction Kit (Beyotime, Nantong), according to the manufacturer’s instructions, and protein concentration was determined using an Enhanced BCA Protein Assay Kit (Beyotime, Nantong). The extracted membrane protein was denatured with 5×SDS loading buffer at 95°C for 5 min and stored at −80°C until analysis. 40 μg denatured proteins were separated through a 12% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (Boster, Wuhan). After incubating in 5% nonfat dried milk for 2 h, the membrane was incubated with 1:10000 HRP-conjugated monoclonal mouse anti-beta actin (Kangcheng, Shanghai) and 1:200 polyclonal GPR41 and GPR43 antibodies (Santa Cruz Biotechnology, Texas) at 4°C overnight. The membrane was washed three times in TBST, and then incubated with 1:10000 diluted horseradish peroxidase-conjugated anti-rabbit antibodies (Sunshibo, Nanjing) for 1 h at room temperature. The membrane was again washed three times in TBST, after which it was incubated in Pierce Western blotting substrate (Pierce Biotechnology, IL) for 1 min. The chemiluminescent signals were visualized by Fujifilm LAS-4000 (Fujifilm, Tokyo).

Immunohistochemistry
The distal ileum, colon, spleen, and adipose tissue were immersed in 4% paraformaldehyde. After fixation, the tissues were washed in 75% alcohol, dehydrated in a graded ethyl alcohol series (85%, 95% I, 95% II, 100% I, and 100% II), cleared in xylene, and embedded in paraffin. The tissues were serially sectioned into 4 μm-thicknesses on a rotary microtome. The paraffin sections were stained using an SABC kit (Boster, Wuhan, China), following the manufacturer’s instructions, and incubated with GPR41 (1:50 diluted) and GPR43 (1:50 diluted) antibody (Santa Cruz Biotechnology, Texas) at 4°C overnight. After immunoreaction, the images were captured on each slide at 400× magnification under a spot camera (Olympus, Tokyo). To check the specificity of the secondary antibody, the sections incubated without the primary antibody were stained by the secondary antibody as a negative control.

Statistical analysis
The real-time PCR data were analyzed using JMP Pro 10. Multiple means were compared using Tukey’s analysis. All the results are expressed as means ± standard deviation (SD). Differences were considered statistically significant at P<0.05, and extremely significant at P<0.01.

Results
Pig chromosome 6 contains GPR41 and GPR43 genes
A search of the pig genome database in GenBank [http://www.ncbi.nlm.nih.gov/genbank/] using the BLAST program [http://blast.ncbi.nlm.nih.gov/Blast.cgi] revealed that pig genome contains GPR41 and GPR43 genes, which are highly similar to these genes in humans, bovines, rats, and mice. The porcine GPR41 and GPR43 genes are located in tandem on chromosome 6. The similarities between porcine GPR41 and human (NM_005306.2), bovine (NM_001163784.1), rat (NM_001005877.1), and mouse (NM_146187.3) were 83%, 83%, 81%, and 84%, respectively.

Full-length amplification and tissue expression of porcine GPR41 and GPR43
Full-length porcine GPR41 was cloned from ileum cDNA, and GPR43 was cloned from spleen cDNA by RACE, as the preliminary RT-PCR results showed that GPR41 and GPR43 were adequately expressed in the ileum and spleen, respectively. Nucleotide sequences of 2218 bp (Accession number: JX566878) and 1908 bp (Accession number: JX566880), representing the full-length cDNA sequences of porcine GPR41 and GPR43, respectively, were obtained. The ORF finder [http://www.ncbi.nlm.nih.gov/orf/index.cgi] was used to predict the open reading frame and the deduced amino acid sequence. The open reading frame of porcine GPR41 and GPR43 was 69–1076 bp and 144–1133 bp, respectively. Porcine GPR41 mRNA was predicted to encode a 335-AA protein (Figure S1), while GPR43 mRNA was predicted to encode a 329-AA protein (Figure S2). The multiple amino acid alignment results among human, bovine, rat, mice, and porcine GPR41 and GPR43 were processed using ClustalW2 multiple sequence alignment [http://www.ebi.ac.uk/Tools/msa/clustalw2/]. The amino acid similarities between porcine GPR41 and human, bovine, rat, and mouse GPR41 were 71%, 82%, 73%, and 76%, respectively (Figure S3). The similarities between porcine and human, bovine, rat, and mouse GPR43 were 82%, 80%, 83%, and 81%, respectively (Figure S4). The analysis results also indicated that these two proteins were membrane proteins containing seven trans-membrane domains.

The pooled RNA was used in this section. The pigs sampled in this study were of the same breed, from different families, of identical age and comparable body weight, and were raised in the same house using the same feed, so that the variation of gene expression would be negligible and using pooled RNA to describe the distribution of GPR41 and GPR43 in various tissues would be acceptable. The RT-PCR analysis indicated that both GPR41 and GPR43 mRNA were expressed in the tested tissues, including liver, spleen, ileum, colon, kidney, adipose tissue, heart, and skeletal muscle (Figure 1).

Expression level of porcine GPR41 and GPR43 in different tissues and different developmental stages
GPR41 and GPR43 were expressed in a significant tissue-specific and time-associated manner (Figures 2 and 3). GPR41 was most adequately expressed in the ileum (Figure 2), which had a significantly higher expression level than any other tested tissue (P<0.01). Its expression level was higher in the spleen than in adipose tissue (P<0.05), and comparable in the liver, colon, and adipose tissue (P>0.05). The highest mRNA level of GPR43 was in the spleen, which had a much higher expression level than the other tissues (P<0.01). There were no differences in GPR43 expression level among the liver, ileum, colon, and adipose tissue (P>0.05).

Porcine GPR41 and GPR43 were also differentially expressed in different developmental stages (Figure 3). The highest expression levels of GPR41 in the liver and colon were on the postnatal day, significantly higher than in the other developmental stages (P<0.05). After birth, the expression levels of GPR41 in the liver and colon were down-regulated. The expression levels of GPR41 in the spleen, ileum, and adipose tissue were up-regulated after birth, with peaks at 70 d in the spleen and ileum and 25 d in adipose tissue. The expression pattern of GPR43 at different developmental
Detection of porcine GPR41 and GPR43 protein by western blot and immunohistochemistry

The tissue distribution of porcine GPR41 and GPR43 proteins was analyzed by western blot (Figure 4). The theoretical molecular weight of porcine GPR41 and GPR43 is about 40 kD; there were weak bands near 40 kD, but the predominant immunoreactive bands were located near 55 kD. Therefore, we considered that the porcine GPR41 and GPR43 proteins might be modified after translation, such as glycosylation and phosphorylation, which could result in a higher molecular weight than theoretical weight. NetPhos 2.0 Server [http://www.cbs.dtu.dk/services/NetPhos/] and NetOGlyc 3.1 Server [http://www.cbs.dtu.dk/services/NetOGlyc/] were utilized to analyze the phosphorylation and glycosylation sites of porcine GPR41 and GPR43. The results showed that GPR41 had 11 potential phosphorylation sites and one O-glycosylation site (Figure S1), while GPR43 had nine potential phosphorylation sites and three O-glycosylation sites (Figure S2).

To identify the cellular distribution of GPR41 and GPR43 in porcine tissues, immunohistochemical staining was performed using GPR41 and GPR43 antibodies. GPR41- and GPR43-immunoreactivities were observed in the spleen, ileum, colon, and adipose tissue (Figure 3), indicating that the cells in these tissues expressed GPR41 and GPR43 proteins.

Discussion

As is known, SCFAs are generated by gut microbial fermentation of complex carbohydrates in the porcine distal small intestine and large intestine [19]. Acetate, propionate and butyrate are predominant SCFAs in the gut lumen of swine [19]. In addition to acting as the substrate for energy generation, SCFAs act as signal molecules and play a regulatory role in a variety of physiological processes. Given their critical regulatory roles, SCFAs and their regulatory functions have drawn much attention. However, the underlying mechanism of SCFAs remains unclear. The identification of SCFA receptors, GPR41 and GPR43, might clarify the mechanism of SCFAs in various physiological processes.

GPR41 and GPR43 were firstly cloned by Sawzdargo et al. [20] in their search for the human galanin receptor subtypes. The GPR40 family, including GPR40, GPR41, GPR42, and GPR43, was found to be located in tandem, downstream from CD22 in chromosome 19q13.1 in humans [20]. Until 2003, three research groups had identified SCFAs as endogenous ligands for GPR41 and GPR43 [11–13]. Subsequent studies identified GPR41 and GPR43 in various tissues of several species, including humans [21,22], rats [23], and bovines [24]. Our study focused on the identification of porcine GPR41 and GPR43 and their tissue distribution.

In the present study, a search of GenBank revealed that the pig genome contains GPR41 (Accession number: XM_003127053.2) and GPR43 (Accession number: XM_003127046.1) genes located in tandem in chromosome 6, and that they are highly similar to those of humans, bovines, rats, and mice. Some of the similarities among these genes were over 80%. However, most of the GPR41 and GPR43 mRNA sequences in GenBank are from a computational prediction, and the organization of GPR41 and GPR43 genes had not been carefully characterized in most of the species. The
amino acid sequences of porcine GPR41 and GPR43 were deduced based on the sequences; these two nucleotide sequences encoded 335-AA and 329-AA protein, respectively. Multiple alignments showed that porcine GPR41 and GPR43 proteins are highly similar to those proteins in humans, bovines, rats, and mice. The structural prediction indicated that these two proteins were membrane proteins containing seven putative trans-membrane domains. However, the functions of porcine GPR41 and GPR43 are poorly understood, and subsequent research will focus on their functions in a variety of physiological processes.

RT-PCR results indicated that GPR41 and GPR43 were expressed in a variety of porcine tissues, including the spleen, ileum, colon, and adipose tissue. However, GPR41 expression in adipose tissue is controversial. Le Poul et al. [12] detected GPR41 expression in human adipose tissue, and Xiong et al. [7] observed that GPR41 was expressed in mouse white adipose tissue and differentiated Ob-luc cells, but not in brown adipose tissue and undifferentiated Ob-luc cells. Conversely, Hong et al. [6] and Wang et al. [24] detected no expression of GPR41 in adipose tissue, even though Hong et al. [6] used the same primers as Xiong et al. [7]. The reason for this discrepancy remains unclear; the inconsistent results might be due to differences in sample origins and techniques. The RT-PCR results identified function genes on the mRNA level, but whether these genes play their role is determined by the translated proteins. Therefore, in this study, we characterized porcine GPR41 and GPR43 by western blot. The western blot analysis showed that the predominant bands for porcine GPR41 and GPR43 were higher than the theoretical molecular weights, suggesting that these two proteins might be modified after translation. The porcine GPR41 and GPR43 amino acid sequences had several potential phosphorylation and glycosylation sites, which were analyzed by online software. The results indicated that porcine GPR41 and GPR43 proteins might be modified after translation and result in target bands with higher molecular than theoretical weights as indicated by western blotting. Tazoe et al. [22] also detected human GPR41 protein near 53 kD by western blotting. When PNGase was used to treat human GPR41 protein, the protein shifted to a lower molecular weight, about 50 kD, indicating that it was indeed glycosylated [22]. However, 50 kD was also higher than the theoretical molecular weight, indicating that other unknown modifications might exist.

In our in situ immunohistochemical study, GPR41 and GPR43 immunoreactivity cells were detected in all tested tissues (spleen, ileum, colon, and adipose tissue). These results were partly in line with published results in humans and rats, in which GPR41 and GPR43 were co-localized with 5-HT- and PYY-containing enteroendocrine cells in the ileum and the colon [21–23]. However, we were unable to confirm the type of GPR41- and GPR43-immunoreactivity cells by the present staining.

Our quantitative real-time PCR results revealed the highest expression levels of porcine GPR41 in the ileum and of GPR43 in the spleen. Tazoe et al. [22] reported that GPR41 was localized in human colon epithelial cells and PYY-containing enteroendocrine cells. PYY, which mediates SCFAs in the inhibition of upper gastrointestinal motility, is released by L cells in the mucosa of the gastrointestinal tract, especially in the ileum and the colon [25]. PYY is also known to be an important appetite control hormone, inhibiting food intake by means of a satiety signal [26]. In this study, porcine GPR41 was most highly expressed in the ileum, suggesting that it might mediate the effects of SCFAs on PYY secretion and PYY-regulated functions. However, whether porcine GPR41 is co-localized with PYY-containing enteroendocrine cells was not directly proved by our immunohistochemistry assay; further research is needed to clarify this question. Porcine GPR43 was adequately expressed in the spleen, suggesting that GPR43 might be implicated in host immune function. Brown et al. [27] also reported the highest expression levels of GPR43 in the spleen in humans and rodents. Maslowski et al. [28] investigated the functions of GPR43 in host immune response and proposed that the SCFA–GPR43 interactions might represent a central mechanism to account for the effects of diet and gut microflora on immune responses and that they may represent new avenues for understanding and potentially manipulating immune responses.

The qPCR results also showed that the expression levels of GPR41 and GPR43 varied at different developmental stages of the tested tissues. The development of porcine gut microbiota and the SCFA-producing capacity at different ages might cause this time-associated manner of expression. The concentration of SCFAs in the gut lumen is quite low at birth, due to the low density of gut microbiota. Afterwards, the gastrointestinal tract is colonized by a variety of bacteria, including Lactobacilli, Streptococci, and Enterobacteria [29]. The abundant diversity and high population of gut microbiota induces a great deal of SCFA generation, which may up-regulate their receptors’ expression. However, in most of the tested tissues, the expression levels of GPR41 and GPR43 were down-regulated in the growing and finishing phases. This finding might indicate that porcine GPR41 and GPR43 mainly exert their functions in the early developmental stages, especially before 70 days. Hong et al. [6] proved that GPR43 is directly involved in adipocyte differentiation in vitro by observing the up-regulated expression levels of GPR43 mRNA in differentiated adipocytes, with a peak at seven days, accompanied by an increase in PPARγ2 and leptin expression. The growth and development of adipose tissue include adipocyte differentiation and hypertrophy. Between 1 and 2 months of age, the increase in adipose tissue was primarily due to the introduction of new adipose cells, while between 3 and 6.5 months, the increase in adipose tissue was mainly due to the

Figure 3. The expression level of GPR41 (left) and GPR43 (right) in liver, spleen, ileum, colon and adipose tissue at different development stages (0, 25, 35, 70, 115, 160 day) of pig (n = 3). The vertical axis of the figure was presented as the target genes copies (GPR41 and GPR43) per 1 microgram total RNA. Data was presented as mean ± SEM. Comparisons were made between different developmental stages in each tissue (*) P<0.05; ** P<0.01.

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Figure 4. Analysis of GPR41 and GPR43 in porcine different tissues by western blot using GPR41 and 43 antibodies. β-actin was used as the loading control. M: marker; L: liver; S: spleen; I: ileum; C: colon; H: heart; K: kidney; AT: adipose tissue; SM: skeletal muscle.

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increase in cell size [30]. Our results showed that the levels of GPR41 and GPR43 mRNA were higher between 25 d and 70 d, indicating that porcine GPR41 and GPR43 might play a critical role in adipocyte differentiation. However, Hou et al. [31] observed that the expression level of GPR43 in adipose tissue of Guangzhong black pigs up-regulated with age, and that the expression level at 10 months was significantly higher than at 2 and 5 months. The difference in the breed of experimental animals (Guangzhong black pig is a fatty-type Chinese pig with a strong capability for depositing fat) might account for the discrepancy. Further studies are necessary to clarify their function in porcine lipid metabolism.

In summary, our study has shown that the pig genome encodes GPR41 and GPR43 genes, and that these two genes are expressed in a variety of porcine tissues, including the spleen, ileum, colon, and adipose tissue. The expression of GPR41 and GPR43 occurs in a significant tissue-specific and time-associated manner, suggesting that these two receptors may have different functions in different tissues and at different developmental stages. The SCFA–GPR41 and –GPR43 interactions might also represent a novel link between gut microbiota and physiological processes. However, further research is required to determine the precise mechanisms of action of GPR41 and GPR43 in various physiological pathways.

**Supporting Information**

**Figure S1** Nucleotide and deduced amino acid sequences of porcine GPR41. The full-length of porcine GPR41 was a 2218 bp nucleotide sequence (Accession number: JX566878), amplified from ileum cDNA, which encoded a 335- AA protein. The protein coding region was 69–1076 bp. Porcine GPR41 protein had seven putative trans-membrane protein and these seven trans-membrane domains were shadowed in this figure. The amino acids labeled with square icons are the potential phosphorylation sites, and labeled with circle icons are the potential Glycosylation sites.

**Figure S2** Nucleotide and deduced amino acid sequences of porcine GPR43. The full-length of porcine GPR43 was a 1908 bp nucleotide sequence (Accession number: JX566880), amplified from spleen cDNA, which encoded a 329- AA protein. The protein coding region was 144–1133 bp. Porcine GPR43 protein had seven putative trans-membrane protein and these seven trans-membrane domains were shadowed in this figure. The amino acids labeled with square icons are the potential phosphorylation sites, and labeled with circle icons are the potential Glycosylation sites.

**Figure S3** Multiple alignments of porcine GPR41 amino acid sequences (Accession number: AFV50552.1) with other known GPR41 (human, bovine, rat and mouse). The GenBank accession number for the protein of human, bovine, rat and mouse are AA13696.1, DAA19942.1, NP_001102382.1 and AA25010.1, respectively.
Multiple alignments of porcine GPR43 amino acid sequences (Accession number: AFV50533.1) with other known GPR43 (human, bovine, rat and mouse). The GenBank accession number for protein of human, bovine, rat and mouse are AAH62006.1, DAA19940.1, NP_001005077.1 and AAH19570.1, respectively.

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Author Contributions
Conceived and designed the experiments: WY GL. Performed the experiments: GL HS ZZ. Analyzed the data: GL. Wrote the paper: GL WY.

References
1. McBumney MI, Sauer WC (1993) Fiber and large bowel energy absorption: validation of the integrated ileostomy-fermentation model using pigs. J Nutr 123: 721–727.
2. Yen JT, Nienaber JA, Hill DA, Pond WG (1991) Potential contribution of absorbed volatile fatty acids to whole-animal energy requirement in conscious swine. J Anim Sci 69: 2001–2012.
3. Bugaut M, Benetjac M (1993) Biological effects of short-chain fatty acids in nonruminant mammals. Annu Rev Nutr 13: 217–241.
4. Farningham DA, Whyte CC (1993) The role of propionate and acetate in the control of food intake in sheep. Brit J Nutr 70: 37–46.
5. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K (2010) Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochemica et Biophysica Acta 1801: 1175–1183.
6. Hong YH, Nishimura Y, Hishikawa D, Tsutsui H, Miyahara H, et al. (2005) Acetate and propionate short chain fatty acids stimulate adipogenesis through GPCR43. Endocrinology 146: 5092–5099.
7. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, et al. (2004) Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. P Natl Acad Sci USA 101: 1045–1050.
8. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ (2006) Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol 40: 235–245.
9. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K (2010) Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochemica et Biophysica Acta 1801: 1175–1183.
10. Bourdu S, Dapoigny M, Roelofsen H, Vonk RJ, Venema K (2010) Identification of a free fatty acid receptor, GPR41. P Natl Acad Sci USA 100: 10361–10366.
11. Scheppach W, Weiler F (2004) The butyrate story: old wine in new bottles? Curr Opin Clin Nutr Metab Care 7: 1–8.
12. Karaki S, Taee H, Hayashi H, Kashiwabara H, Tooyama K, et al. (2008) Expression of the short-chain fatty acid receptor, GPR43, in the human colon. J Mol Histol 39: 235–242.
13. Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, et al. (2006) Expression of short-chain fatty acid receptor GPR41 in the human colon. Biochem Biophys Res Commun 299: 231–236.
14. Dewulf EM, Cani PD, Neyrinck AM, Possemiers S, Van Holle A, et al. (2011) 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 22: 712–722.
15. Bjorell M, Admyre T, Goransson M, Marley AE, Smith DM, et al. (2011) Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. AM J Physiol-Endoc Met 300: E211–E220.
16. Gilbert ER, Li H, Emmerson DA, Webb KE, Wong EA (2008) Dietary protein quality and feed restriction influence abundance of nutrient transporter mRNA in the small intestine of broiler chicks. J Nutr 138: 262–271.
17. Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70: 567–590.
18. Sawdzardo M, George SR, Nguyen T, Xu S, Kolakowski LF, et al. (1997) A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1. Biochem Biophys Res Commun 239: 343–347.
19. Karaki S, Taee H, Hayashi H, Kashiwabara H, Tooyama K, et al. (2008) Expression of the short-chain fatty acid receptor, GPR43, in the human colon. J Mol Histol 39: 135–142.
20. Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, et al. (2006) Short-chain fatty acid receptor, GPR41, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 324: 353–360.
21. Wang A, Gu Z, Heid B, Akers RM, Jiang H (2009) Identification and characterization of the bovine G protein-coupled receptor GPR41 and GPR43 genes. J Dairy Sci 92: 2906–2913.
22. Lundberg JM, Tatemoto K, Terenius L, Hellstrom PM, Mutt V, et al. (1982) Localization of peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. P Natl Acad Sci USA 79: 4471–4475.
23. Ween AM, Bloom SR (2007) Gut hormones and appetite control. Gastroenterology 132: 2116–2130.
24. Brown AJ, Jaure S, Briscoe CP (2005) A family of fatty acid binding receptors. DNA Cell Biol 24: 54–61.
25. Maciowska KM, Vieira AT, Ng A, Kranich J, Sierro F, et al. (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR41. Nature 461: 1202–1206.
26. Mackie RI, Sihl A, Gaskin HR (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. AM J Clin Nutr 69: 10358–10458.
27. Anderson DB, Kauffman RG (1973) Cellular and enzymatic changes in porcine neonatal gastrointestinal tract. AM J Clin Nutr 69: 10358–10458.
28. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, et al. (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR41. Nature 461: 1202–1206.
29. Mackie RI, Sihl A, Gaskin HR (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. AM J Clin Nutr 69: 10358–10458.
30. Anderson DB, Kauffman RG (1973) Cellular and enzymatic changes in porcine neonatal gastrointestinal tract. AM J Clin Nutr 69: 10358–10458.
31. Hou Z, Sun C (2008) Transcriptional expression of GPR43 gene in adipose tissue and primary cultured adipocytes of pig. Sheng Wu Gong Ching Xue Bao 24: 1361–1366.