Research Article
Identification of Three Novel Splicing Variants and Expression Analysis of Chicken GPR1 Gene

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GPR1 is a G protein-coupled receptor that plays critical roles in eukaryotic cells: typically, response to glucose stimulation, lipid accumulation, and transmitting nutrition signals to cAMP pathway. However, the alternative splicing of the GPR1 gene and its expression pattern in chicken tissues and ovarian follicles were unknown. In our current study, we used RACE-PCR to identify three GPR1 variants, including the full-length variant (GPR1-va1) and two alternatively spliced variants (GPR1-va2, GPR1-vb). Quantitative real-time PCR examined the expression pattern of GPR1 mRNA in chicken tissues and ovarian follicles. The result reveals that the coding sequence of the three variants cDNA is 1053, 1053, and 627bp in length, encoding 350, 350, and 208 amino acids, respectively. The three variants of GPR1 show similar tissue distributions; GPR1 expression was abundant in the abdominal fat, lung, and heart. With the follicular development, the expression of GPR1 gene gradually increased, and GPR1-va1 and GPR1-va2 spliced variants expression in F2 were significantly higher than in F5, F4, and prehierarchical follicles (P < 0.05). Taken together, we found three novel variants of GPR1, and the results of GPR1 expression profiling in adipose tissues and ovarian follicles suggest that GPR1 may play a significant role in the lipid accumulation and progression of follicular development.

1. Introduction

Many signaling transductions are mediated by G protein-coupled receptors (GPCRs) in eukaryon [1]. G protein-coupled receptors exist in eukaryotes, including yeast, choanoflagellates, and animals [2]. GPR1 is a G protein-coupled receptor (GPCR), originally found in human [3], which was identified by in vitro experiment as receptor for chemerin [4, 5]. GPR1 and chemerin are related to adipogenesis [6–9], circadian appetite regulation [10], cell chemotaxis [11], inflammation [6, 12, 13], and phosphorylation of ERK and Akt [14].

Alternative splicing (AS) of pre-mRNA can generate diversity form protein subtypes from a single gene [15–17]. In many instances, coding sequence was affected by alternative splicing, which would result in the production of diverse proteins [18]. Various proteins would be produced due to different open reading frames [19]. In some kind of situation, partly different proteins may have various functions, lacking or having a special function [20]. Recent studies using next generation sequencing have demonstrated that AS could generate huge transcriptional isoforms of mammalian gene [16, 21–23]. Alternative splicing has been demonstrated to act as a major mechanism that modulates gene expression and function of GPCRs [24–26].

In this study, we identified three novel GPR1 splice variants. We designated the novel variants GPR1-va1, GPR1-va2, and GPR1-vb. GPR1-va1 and GPR1-va2 and GPR1-vb use the same translation start codon. However, the CDS of GPR1-vb was different from GPR1-va1 and GPR1-va2, and thus as a result they have different amino acid sequences. We examined the different expression profiling between the three variants in tissue and ovary follicles distribution using qRT-PCR. These data could increase our knowledge of GPR1 mRNA diversity and provides the basis for further functional research.
2. Materials and Methods

2.1. Experimental Animals and Tissue Sampling. Three producing female Lohmann pink tissues (Gallus gallus) in the fiftieth week were selected for sampling from the Experimental Farm for Fowl Breeding at Sichuan Agricultural University (Sichuan, China). These chickens were hatched on the same day and grown under the same natural conditions of light and temperature. Eye, brain, hypothalamus, pituitary, ovary, oviduct, adipose tissues, muscle tissues, POF, lung, spleen, kidney, and ovarian and granulosa cells from 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, and 8 to 9 mm diameter prehierarchical follicles, and F5–F1 (measuring F1 >3 to4, 4 to5, 5 to6, 6 to7, 7 to8, and8 to9mm) were selected for sampling from the Experimental Animal Care Guidelines.

2.2. Reverse Transcription PCR. Total RNA was extracted from these samples with TRIzol (TaKaRa, Dalian, China). cDNAs were synthesized using a PrimeScript® RT Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. In brief, step 1: the 10.0 µL reaction consisted of 1.0 µL of total RNA, 2.0 µL of 5x gDNA Eraser Buffer, 1.0 µL of gDNA Eraser, and 5.0 µL of RNase-Free dH2O. Thermal cycling was executed for 2 min at 42°C. Step 2: the 20.0 µL reaction consisted of 10.0 µL of the reaction solution from step 1, 1.0 µL of PrimeScript RT Enzyme Mix I, 1.0 µL of RT Primer Mix, 4.0 µL of 5x PrimeScript Buffer 2 (for Real Time), and 4.0 µL of RNase-Free dH2O. Thermal cycling was executed for 15 min at 37°C and then 5 sec at 85°C.

2.3. 5'-/3'-Rapid Amplification of cDNA Ends-PCR. Total RNA was extracted from mix sample (hypothalamus, pituitary, oviduct, adipose tissues, and muscle tissues) with RNeasy Mini Kit (Qiagen, German) and subsequently processed with the SMART-rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech, USA). RACE-PCR were carried out using 1.5 µL of 5-fold diluted 3'-RACE (or 5'-RACE)-ready cDNA as template in a 50 µL reaction under the following cycling conditions: The first-round PCR: 94°C for 3 min; 5 cycles at 94°C for 30 s and 72°C for 4 min; 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 4 min; 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 4 min; the second-round PCR: 94°C for 3 min and 16 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 4 min; the third-round PCR: 94°C for 3 min and 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 4 min. Detailed information on the RACE-PCR primers (1.1–3.3) is provided in Table 1.

2.4. Quantitative Real-Time PCR. Total RNA was isolated from these samples of each hen with TRIzol reagent (TaKaRa, Dalian, China). Approximately 1 µg of DNase-treated RNA from each sample was reverse transcribed with a cDNA Synthesis Kit. The cDNA samples were diluted 4-fold and subjected to qRT-PCR on a C1000™ Thermal Cycler (Bio-Rad, CA, USA). Each qRT-PCR was performed in a 25 µL volume containing 1.5 µL of diluted cDNA, 12.5 µL of 2x SYBR Premix Ex-Taq II (TaKaRa, Dalian, China), and 1.2 µL of variant-specific primer pair mix (10 pmol/µL each primer). All variant-specific primer pairs were run with the same cycling conditions: 95°C for 30 s followed by 46 cycles of 95°C for 5 s and 60°C for 30 s with a final melting curve analysis (from 65°C to 95°C at a rate of 0.5°C per 5 s). The melting curve analyses showed that the amplification efficiency of each variant-specific primer pair was higher than 97%. Negative and positive controls were included in each experiment as quality control and threshold cycle (Ct) calibration steps.

The expression levels of the target genes were calculated using geNORM algorithms [27] based on the geometric means of two reference genes: β-actin and GAPDH. Each sample was run in triplicate.

Detailed information on the qRT-PCR primers (4.1–7.2) is provided in Table 1.
### Table 1: Primers used to study GPR1.

| S. number | Primer name | Sequence (5'- to 3'-end) | Source | Purpose |
|-----------|-------------|---------------------------|--------|---------|
| 1.1       | GRI-5'-1    | CAGATGACAATGGCATGCTGGGACTC | Conserved domains of published sequences (XM_015289515.1, 971405019) | To obtain 5'-UTR and 3'-UTR |
| 1.2       | GRI-5'-2    | TATGGGCAATGCTGAGGGTGACIGG |        |         |
| 1.3       | GRI-5'-3    | CTTCCTCAGGCCAGTCATAGAAATAAGAGTAG |        |         |
| 2.1       | UPM-long   | ATAGGGCAAGCAGTGATCTACGAGCAGT |        |         |
| 2.2       | UPM-short  | ATAGGGCCAGCAGTGATCTACGAGCAGT | Conserved domains of published sequences (XM_015289515.1, 971405019) | To obtain 5'-UTR and 3'-UTR |
| 3.1       | GRI-3'-1    | GAGGAAGGCAGTACCAAGCTACGAGCAGTG |        |         |
| 3.2       | GRI-3'-2    | CACACTGAACTGACACTGATACGAGCAGTG |        |         |
| 3.3       | GRI-3'-3    | GCTACAGAGGACACATGCCGACTACGAGTGT |        |         |
| 4.1       | GPR1-va-F   | TGACTGTTTACCATCCATCCATCT |        | To measure expression levels of GPR1-va and GPR1-vb by qRT-PCR, respectively |
| 4.2       | GPR1-va-R   | ATGCTGCAACCCGACACACT |        |         |
| 5.1       | GPR1-vb-F   | TGTCACATTTGCTACAAATGAC |        |         |
| 5.2       | GPR1-vb-R   | ATGCTGCAACCCGACACACT |        |         |
| 6.1       | β-Actin-F2  | TGCTGCTCCCAGTGATGCCCT | Specific regions of *Gallus gallus* β-actin (L08165.1) |         |
| 6.2       | β-Actin-R2  | GGAGGGCTGACCATCATTGACTGACTGAGTC |        |         |
| 7.1       | GAPDH-F     | CCAGAACACATCACCAGGCTG | Specific regions of *Gallus gallus* GAPDH (NM_204305.1) |         |
| 7.2       | GAPDH-R     | ACGGCAGGCCGAGTCACAA |        |         |
Figure 1: Gel electrophoresis images of GPR1 RACE-PCR products. PCR products were amplified with nested PCR. Gel pictures analysis suggesting the presence of multiple GPR1 variants. Note a single band in 5′-RACE-PCR and multiple amplicons in 3′-RACE-PCR. The PCR products of GPR1 were separated on 1% agarose gel following electrophoresis and visualized with ethidium bromide. MTs” represents mixtures of cDNA (hypothalamus, pituitary, oviduct, adipose tissues, and muscle tissues). Round 1, Round 2, and Round 3 represent the first-round PCR, the second-round PCR, and the third-round PCR, respectively.

Table 2: Sequence analysis of three GPR1 variants.

| Isoform     | Total length (nt) | 5′-UTR length (nt) | CDS length (nt) | 3′-UTR length (nt) | Poly(A) length (nt) |
|-------------|-------------------|--------------------|-----------------|-------------------|---------------------|
| GPR1-val    | 2875              | 160                | 1053            | 1634              | 28                  |
| GPR1-va2    | 2377              | 160                | 1053            | 1139              | 25                  |
| GPR1-vb     | 2343              | 160                | 627             | 1528              | 28                  |

2.7. Statistical Analysis. All data were analyzed by a one-way analysis of variance (ANOVA), which was followed by Duncan’s multiple range test, using the SAS 9.0 statistical software for Windows (SAS Institute Inc., USA). Values were expressed as the mean ± SEM, n = 3. Differences were considered significant at P < 0.05.

3. Results

3.1. Analysis of GPR1 Variants Sequence Characteristics

3.1.1. Multiple GPR1 Variants. To investigate chicken GPR1, we cloned splice variants of GPR1 by RACE-PCR. We obtained three full-length mRNA sequences alternative splice variants (Figure 1). Similarity analysis identified three GPR1 variants: GPR1-val (KX156840), GPR1-va2 (KX156841), and GPR1-vb (KX156842).

BLASTn alignments showed that although all variants are most similar to vertebrate GPR1, GPR1-val shows the highest similarity to GPR1 mRNA, with 99%, 94%, 93%, and 86% similarity to Meleagris gallopavo, Anser cygnoides, Anas platyrhynchos, and Coturnix Japonica, respectively. In addition, MegAlign analysis suggests that they are all splice variants.

Phylogenetic tree analysis using GPR1-val sequences from other vertebrate species has shown that the chicken GPR1-val is most closely related to the GPR1 sequence in Meleagris gallopavo followed by those in Anser cygnoides and Taeniopygia guttata (Figure 2).

3.1.2. Structural Analysis of GPR1 Variants. Spidey analysis revealed that GPR1 comprises two exons and one intron (Figure 4(a)). All three GPR1 variants are generated from a single sequence through different splicing modes (Figure 4(b)). In addition, all splicing modes are consistent with the canonical 5′-GU—AG 3′-donor—acceptor splice site pairs rule. ORF Finder and Spidey analysis of the three GPR1 variants showed that although each variant has an identical short 5′-UTR (untranslated region), the CDS and 3′-UTRs vary significantly in size, ranging from 627 to 1053 bp and 1139 to 1634 bp, respectively (Figure 4(b) and Table 2).

1-TASSER (Figure 4(c)), Rosetta (supplementary Fig. 1B, in Supplementary Material available online at https://doi.org/10.1155/2017/1074054), TMHMM, and DNAMAN (Figure 3) comparison of the putative GPR1 receptors encoded by these variants revealed that GPR1 exhibits the typical type A GPCR features including seven transmembrane a-helical
domains connected by three extracellular and three intracellular loops. However, GPR1-vb variants were all truncated proteins, with partial transmembrane domains from type A GPCRs. GPR1-vb spanned 627 bp and encoded 208 amino acids, which share 90.4% similarity with GPR1-va (GPR1-va1 and GPR1-va2 contain the same amino acid sequence).

3.2. Expression Analysis of GPR1 Variants mRNA in Lohmann Pink Tissues. qRT-PCR analysis showed GPR1 variants mRNA expression in all tissues (Figure 5). The highest expression level of GPR1-va (GPR1-va1 and GPR1-va2 have the same coding sequence) in the Lohmann pink tissues was detected in the abdominal fat and lung (P < 0.05). GPR1-vb mRNA expression in the abdominal fat was also significantly high compared to other tissues (P < 0.05). In contrast, the lowest expression level was observed in the hypothalamus (P < 0.05).

qRT-PCR revealed GPR1 variants expression in different follicles of the Lohmann pink ovary (Figure 6). GPR1 transcripts were detected in all experimental hierarchical follicles.
and prehierarchical follicles of the Lohmann pink ovary. With follicular development, GPR1-va expression levels gradually increased, with levels in F2 significantly higher than that in F4, F5, and prehierarchical follicles (P < 0.05). There was also a high expression of GPR1-va in the F2 compared to 1 to 2, 2 to 3, and 3 to 4 mm diameter prehierarchical follicles (P < 0.05). The lowest GPR1-va and GPR1-vb gene expression were detected in 1 to 2 mm diameter prehierarchical follicles (1-2 mm).

4. Discussion

Previous studies on GPR1 mainly used rodent animal models and little was known about the molecular characteristics in aves such as chicken. In this study, we identified three novel GPR1 splice variants that have partially different CDS and 3′-UTR from the GPR1 originally reported. GPR1-va1, GPR1-va2, and GPR1-vb are alternatively spliced variants. mRNA for GPR1-va1 uses exon 1 and exon 2, mRNA for GPR1-va2 lacks a segment sequence in 3′-UTR (Figure 4(b)), and mRNA for GPR1-vb lacks a segment sequence in CDS (Figure 4(b)). Interestingly, we compared the forecasting method of I-TASSER and Rosetta, and we find some differences in 3D structure (Figure 4(c) and supplementary Fig. 1B). Perhaps the main reason is that I-TASSER and Rosetta adopted TBM+FM and FM forecasting method, respectively [33]. The differently three-dimensional structure of the protein determines the different function of protein; therefore, improving the performance of protein structure prediction algorithm is a key technique in the further study. To date, many GPCRs splice variants have been reported invertebrates [26, 34–36]. Alternative splicing (AS) that generates complexity before mRNA can produce distinct mRNA and protein isoforms.
GPR1 is ubiquitously expressed in most tissues, and GPR1 expression profile is the same as swine [6] and mouse [37, 38]. AS could result in physiological diversity such as differences in tissue distribution, ligand-binding properties, signaling pathways, and coupling efficiency with Gα protein [39]. The tissues distributed of GPR1-va (GPR1-va1 and GPR1-va2) is similar with GPR1-vb. But GPR1-va was more abundantly expressed (Figures 5 and 6).

Because GPR1-va1, GPR1-va2, and GPR-vb use the same transcription initiation site, the ratio of mRNAs for GPR1-va1, GPR1-va2, and GPR-vb may be determined by post-transcriptional regulation, such as splicing efficiency and mRNA stability [40, 41]. The stability of the mRNA may be different between GPR1-va1, GPR1-va2, and GPR-vb. Splicing is regulated by several factors such as splice site recognition, splicing regulators, and RNA secondary structure [42–44]. In vertebrate genes, splice sites are not well conserved, which allows alternative splicing to occur frequently [45]. If introns are retained within the CDS, the site of a stop codon would contribute to the production of a truncated peptide (if inserted close to the 3'-end) or the absence of a protein product (if inserted downstream of the start codon) [46]. In our study, the stop codon of GPR1-vb was not changed relative to GPR1-va1 and GPR1-va2; we found that the translatability of about 426 nucleotides might indeed be interrupted by the retention of an intron in the CDS. This type of event was typically disregarded owing to the absence of protein products; however, intron retention might also contribute to the diversification of the information carried by genes, by producing functional RNA [47].

GPR1 is ubiquitously expressed in most tissues, and GPR1 expression profile is the same as swine [6] and mouse.
In our study, GPR1-va and GPR-vb mRNA were expressed in all tissues examined and in a highly tissue-specific manner in the Lohmann pink tissues. The high levels of GPR1-va and GPR-vb were detected in the adipose tissue which implicates its potential key role in regulating chicken adipocyte development. In addition, GPR1-va and GPR-vb mRNA were expressed at the highest levels in the abdominal fat, followed by muscle, lung, subcutaneous fat, heart, eye, and other tissues, which confirmed GPR1 mRNA tissue-specific expression in different chicken tissues. This result is coincident with previous studies in mouse (gender and age not indicated, obese mice) [10, 49]. However, the highest GPR1 mRNA expression level is found in the kidney of pig (males, 2.5 months old) [6] and in the skeletal muscle of mouse (gender and age not indicated, obese/diabetic mice) [50]. Therefore, we cannot eliminate the possibility that GPR1-va1/GPR1-va2/GPR-vb shows different expression patterns in species-, gender-, or temporal-specific profiles or that it shows different functions within the adipose tissue.

In our current study, GPR1-va1, GPR1-va2, and GPR-vb expression gradually increased with follicular development, suggesting that GPR1-va1, GPR1-va2, and GPR-vb may regulate follicular development in the Lohmann pink tissues. However, the level of GPR1-va1, GPR1-va2, and GPR-vb expression was highest in the F2 than in the other follicles (prehierarchical follicles, F5, F4, F3, F1, and POF). Previous studies showed that the high level of GPR1 was detected in subcutaneous fat [6], which speculated that GPR1 could play a role in lipid accumulation. Moreover, lipid and lipid metabolism play a crucial role in cell survival and proliferation [51, 52]. In addition, GPR1 and CMKLR1 are the coreceptors for chemerin, and they have the closest phylogenetic relationship in the family of chemoattractant receptors [6, 53]. Goralski et al. study suggested that chemerin and CMKLR1 could regulate adipogenesis and adipocyte metabolism through ERK1/2 signaling pathway [54]. Various follicle classes have different lipid characteristics [55]; by adding GPR1 antibody and PI3K signaling inhibitor we find that the chemerin/GPR1 and PI3K signaling pathways may be involved in follicular development [56]. Therefore, GPR1 is the only known receptor for chemerin which also may regulate the follicular development through regulating follicular lipid accumulation. In this study, expression of GPR1 showed an increased tendency gradually with follicular growth; however, the expression of GPR1 tends to decrease in the F1, suggesting that follicular internal environment may be changed such as lipid metabolism. Interestingly, study on GPR1 KO mice speculated that CMKLR1 compensated for the loss of GPR1 function [10]. As such future studies examine follicular development and lipid metabolism through both CMKLR1 and GPR1 when investigating the signal transduction mechanisms of chemerin function.

5. Conclusion

In this study, we cloned three alternative splice variants of GPR1 full-length mRNA sequences from Lohmann pink tissues. The GPR1 transcript was widely distributed in various tissues. With follicular development, GPR1 gene expression gradually increased; GPR1-va expression in F2 was significantly higher than in F5. The results of the GPR1 expression profiling of ovarian follicles suggested that GPR1 plays key role in follicular development through regulating the lipid levels. Therefore, our findings increase our knowledge of GPR1 mRNA diversity and provide a solid basis for further molecular mechanism research.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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