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Molecular Cloning, Sequence Analysis and Tissue Expression of Porcine *R-Spondin2*

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Abstract

The R-spondin 2 (Rspo2) belongs to the Rspo family, which is a cluster of secreted molecules possessing pleiotropic functions in development and stem cell growth by strongly enhancing Wnt signaling activation. In this study, porcine *Rspo2 (pRspo2)* was amplified from cDNA derived from porcine brain tissue. DNA sequencing result indicates that the open reading frame (ORF) of pRspo2 consists of 732 base pairs (bp), encodes 243 amino acids protein with a putative molecular weight of 28.19 kDa and is high identity to those of horse (98%), cattle (98%), human (96%) and rat (92%). pRspo2 is highly expressed in the brain tissue, whereas with comparatively lower level of expression in duodenum, thymus, liver, lung, lymph node, kidney and ileum tissues, which is characterized by reverse transcription-polymerase chain reaction (RT-PCR) analysis. All the results above will provide the fundamental research on the pleiotropic functions of *pRspo2*.

Keywords: *Rspo2*; Cloning; Expression; Porcine

Introduction

R-Spondin (Rspo) proteins are a cluster of secreted molecules which strongly potentiate Wnt/β-catenin signaling. There are four members of the Rspo family in vertebrates (Rspo1-4), and all of them can activate Wnt signaling pathway [1]. Rspos own pleiotropic functions in development and stem cell growth by strongly enhancing Wnt pathway activation [2-6]. These proteins contain two N-terminal furin-type, cysteine-rich domains, one C-terminal thrombospondin domain. Studies have suggested that the furin-type domains are responsible for the activation of Wnt/β-catenin signaling [7].

The functional linkage between Rspos and Wnt signaling has been established by the identification of Xenopus Rspo2 as a novel activator of Wnt/β-catenin signaling [7]. Rspo2 also appears to play an indispensable role in muscle development in mouse, as well as in Xenopus embryos [8,9]. Since *Rspo2*$^{-/-}$ mice exhibit midfacial skeletal defects, limb loss and lung hypoplasia, it is easy to infer that Rspo2 regulates midfacial, limb, and lung morphogenesis during development through the Wnt/β-catenin signaling [9]. Mutation of Rspo2 leads to the appearance of short hair on the head, face, and lower legs in the Portuguese water dog [7]. In one study of the goat gonads, Rspo2 is considered as a candidate gene for ovarian differentiation [10]. Rspo2 also is a well-established candidate for monitoring of hair-growth phenotype as it synergizes with Wnt to activate β-catenin [11], and Wnt signaling is required for the establishment of the hair follicles [12,13]. The change of 3' untranslated region (3'UTR) causes a threefold increase in Rspo2 transcripts in muzzle skin biopsies of dogs with furnishings, consistent with a transcript effect [14]. As multiple functions of Rspo2 have been previously studied, all of which establish its critical roles in developmental biology. In this study, we cloned pRspo2, analyzed its bioinformatics analysis and detected its mRNA expression levels in different tissues. These results will facilitate the future study of pRspo2 functions.

Materials and Methods

RNA extraction and cDNA synthesis

Tissue samples of the heart, liver, spleen, lung, kidney, jejunum, duodenum, ileum, rectum, brain, lymph node, thymus, uterus and muscle were used as previously described [15]. Total RNA was extracted using a standard Trizol RNA isolation protocol (Invitrogen, USA). The purity and quantity of total RNA were measured with an ultraviolet/visible spectrophotometer (Nanodrop 2000, Wilmington, DE, USA). cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, WI, USA), ~1 μg RNA, and an oligo (dT) 18 primer.

Molecular cloning of *pRspo2*

The reverse transcription polymerase chain reaction (RT-PCR) was performed to isolate pRspo2 using cDNA from porcine brain tissue. The primers were designed based on the open reading frame (ORF) of in silico sequence assembly by Primer Premier TM Version 5.0 software (PREMIER Biosoft International, Canada). The sequence of pRspo2 was confirmed by DNA sequencing. The forward primer (5' –CTAGCTAGCATGCAGTTTCGTCTCTT

CTCC-3') and reverse primer (5'-CCGCTCGAGTTACT-GATTAGCTCTATCTG-3') contain *NheI* and *XhoI* restriction enzyme sites, respectively. The PCR program initially started with a 95°C denaturation for 5 min, followed by 30 cycles of 95°C for 30s, 60°C for 30s and 72°C for 50s, then 72°C extension for 10 min. The PCR product was separated on 1.0% agarose gels and recovered by SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China).

The purified PCR product was cloned into the pMD19-T vector (Takara, Dalian, China). The recombinant plasmids were transformed into competent Escherichia coli strain DH5α. After the blue-white screening, the positive colonies were confirmed by PCR and DNA sequencing (Sangon Biotech, Shanghai). The complete ORF of *pRspo2* has been deposited into the GenBank database and assigned GenBank accession No.KC989556.

Sequence analysis of porcine *Rspo2*

The cDNA sequence was blasted using BLAST at the web server

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of the National Center of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). The protein prediction and analysis were performed using the BioX 2.6 software. PSIPRED Protein Sequence on-line analysis (http://bioinf.cs.ucl.ac.uk/psipred/) was conducted to predict the secondary structure of the inferred amino acid sequences. Homologous analysis of different species of pRspo2 was performed with DNAMAN software. TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to predict the transmembrane domain of the inferred amino acid sequences. Group-based Prediction System (http://gps.biocuckoo.org/online.php) was used to predict phosphorylation sites of the encoded protein [16].

Tissue expression analysis of *pRspo2*

To determine the expression profiles of pRspo2, RT-PCR was carried out with the primers (5'-TGTGTGGAAGGATGTGAGGT-3'; 5'-CCTTTGCCTTTGGAGCTCTC-3') designed and synthesized based on the alignments of the pRspo2 sequence, resulting in a PCR product of 220 bp. As an internal control, the cDNA from the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified by control primers (5'-TCAAGAAGGGGAACGAGG AC-3'; 5'-TTCTTGGTGCCAGACTTTGC-3') resulting in a 179 bp product. The 20 μl reaction system contained 2.0 μl pooled cDNA for each tissue (100 ng/μl), 0.5 μl forward primer (20 μM), 0.5 μl reverse primer (20 μM), 10 μl Premix Taq DNA polymerase (1.25 U/25 μl; TaKaRa), and 7 μl sterile water. PCR was conducted as follows: 95°C for 5 min; 30 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 20s; 72°C extension for 10 min. The RT-PCR products (10 μl) from each tissue were electrophoresed on 1.5% agarose gels. Gray scanning analysis by Gel-PRO Analyzer software was conducted to normalise the expression level of *pRspo2* in each tissues with GAPDH.

Results

Molecular cloning and nucleotide sequence analysis of *pRspo2*

Based on *in silico* sequence assembly, the pRspo2 gene was identified from porcine brain cDNA and cloned into pMD19-T vector (Figure 1A). After transformation and overnight culture, positive colonies were confirmed by PCR and DNA'sequencing (Figure 1B). The 732 bp ORF of pRspo2 encoded 243 amino acids with an assumed molecular weight of 28.19 kDa, BioX 2.6 software analysis suggested that the isoelectric point was 9.39. The nucleotide and protein sequences of the pRspo2 were shown in (Figure 2). These cDNA nucleotide sequence analysis using the BLAST software at NCBI server showed that the gene was not homologous to any of the known porcine genes and it has been submitted into the GenBank database (GenBank accession No. KC989556).

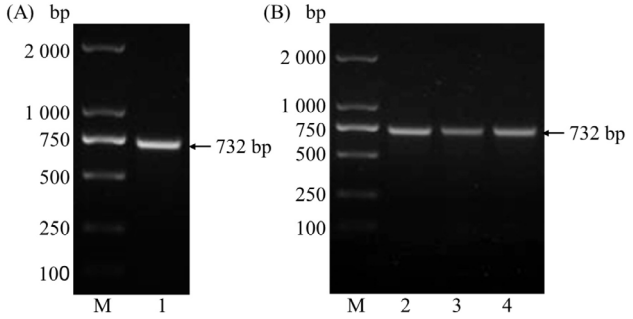

Figure 1: RT-PCR results for pRspo2 (A) and PCR identification of pMD19T-Rspo2 (B). Lane M=DL2000 DNA markers; lane 1=PCR product for pRspo2 (732 bp); lane 2-4=PCR results of pMD19T-Rspo2 by amplification primers (732 bp).

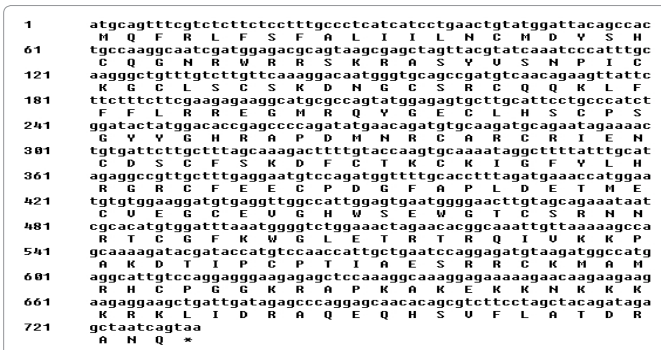

Figure 2: The sequence of *pRspo2* (GenBank accession No. KC989556). The entire deduced amino acid sequence is depicted in single letter code beneath the corresponding nucleotide sequence. The stop codon TAA is with an asterisk.

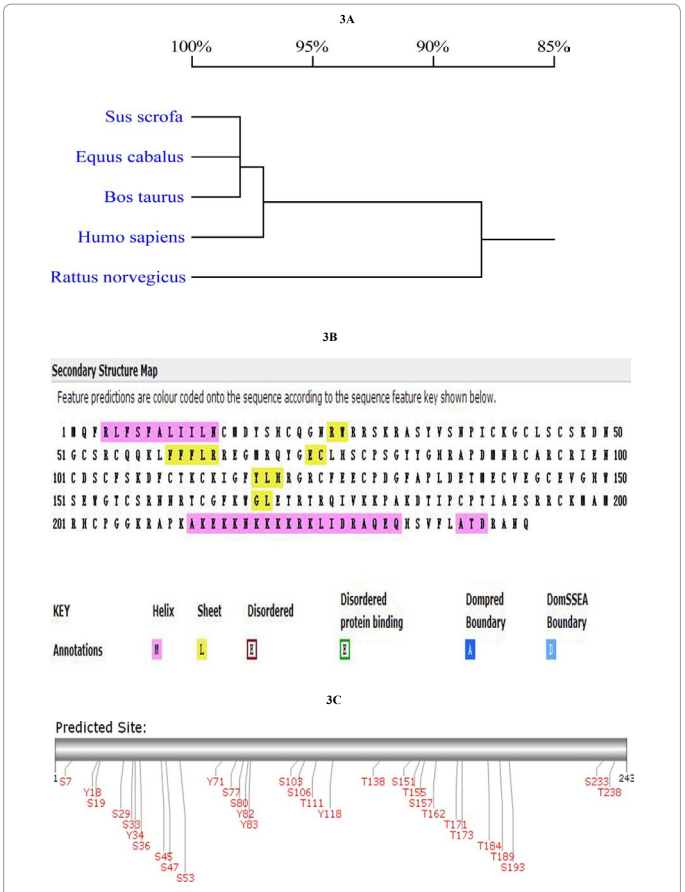

Figure 3: Analysis of the amino acid sequence of pRspo2. (A) Homology alignment of Rspo2 in *Sus scrofa, Equus cabalus, Bos Taurus, Homo sapiens and Rattus norvegicus*. (B) Secondary structure prediction of pRspo2. (C) The positional distribution of predicted kinase-specific phosphorylation sites in pRspo2. S: serines, T: threonines, Y: tyrosines.

Structure and functional prediction of *pRspo2*

The BLAST results showed that the amino acid sequence of pRspo2 has high homology with those of horse (98%), cattle (98%), and human (96%) and rat (92%) (Figure 3A). In order to further investigate the detailed structure of pRspo2, PSIPRED Protein Sequence on-line analysis software was employed to conduct the secondary structure in pRspo2. The result indicated that pRspo2 contained four alpha-helixes, six β-sheets and several random coils (Figure 3B). In addition, the TMHMM result demonstrated that pRspo2 had no evident

transmembrane helices, which signified that pRspo2 was not a Tran's membrane protein. Group-based Prediction System (GSP) results revealed thirty-one phosphorylation sites in pRspo2 (Figure 3C).

Tissue expression analysis of *pRspo2*

The distribution of *pRspo2* mRNA in different tissues was analyzed by RT-PCR. Samples from 14 different tissues from Sanyuan pigs were analyzed in the study. After electrophoresis, gray scanning analysis showed that *pRspo2* was highly expressed in the brain, and was expressed at relative low levels in the duodenum, thymus, liver, lung, lymph node, kidney and ileum. No expression was observed in other tissues (Figure 4).

Discussion

As we all know, the Wnt signaling pathway plays a central role in multiple biological processes during embryonic development, adult homeostasis and disease pathogenesis [17,18]. In the adult organism, Wnt signaling is essentially involved in the homeostasis of many tissues, including the intestine, skin, bone and hematopoietic system [17, 19]. Moreover, Wnt signaling is also essential in stem cell self-renewal [17,19]. The Rspo protein family consists of four secreted proteins (Rspo1-4) that are identified as strong potentiates of Wnt/β-catenin signaling via a common mechanism [1,7,20,21]. However, Rspo2 and Rspo3 are more active than Rspo1 and Rspo4 [2].

In this study, the ORF of *pRspo2* has been cloned, which encodes a protein of 243 amino acids. The BLAST result shows that the amino acid sequence is consistent with the predicted sequence of porcine Rspo2 (XP_003481408). In addition, high homology of pRspo2 suggests that it is orthologous among different vertebrates [2]. The TMHMM result of pRspo2 is consistent with that Rspo2 is a secreting protein [7], which induces Wnt/β-catenin signaling by binding to the extracellular domain of LGR4 and LGR5 [22,23]. Protein phosphorylation is a ubiquitous regulatory switch that plays an important role in the regulation of many signal transduction mechanisms. Active site analysis suggests that the protein kinases acting on pRspo2 are mainly serine/threonine protein kinases, which participate in multiple signaling pathways of cell proliferation, differentiation, inflammation and apoptosis [24].

Studies have showed that Rspo plays an essential role during vertebrate development and is expressed in the endocrine cells of intestine, pancreas and adrenal gland, meanwhile in the brain, gonad, prostate and kidney [25,26]. In this study, the expression of *pRspo2* is relatively high in the brain, and comparative lower in the duodenum, thymus, liver, lung, lymph node, kidney and ileum. The result is basically

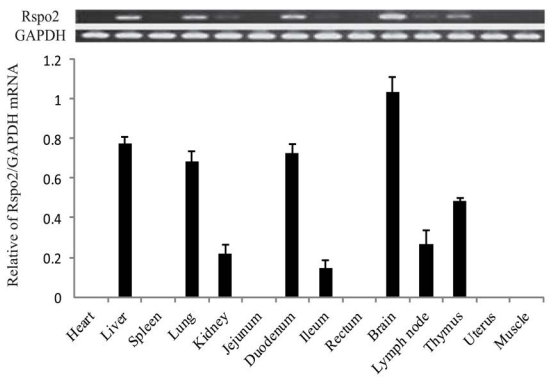

Figure 4: Relative *pRspo2* mRNA expression in different tissues by semi-quantitative RT-PCR analysis. GAPDH was used as the internal control against which the expression of *pRspo2* mRMA was normalized. Error bars represent SD.

similar to that of medaka *Rspo2*, which is ubiquitously expressed in the brain, liver, heart, intestine, kidney, ovary and testis, with dominant expression in the brain, liver and ovary [10]. The high level expression of *pRspo2* in brain indicates that it plays an important role in the brain. Furthermore, *pRspo2* is also expressed abundantly in the lymph node and thymus, suggesting that Rspo2 may have immunological activity or participate in the development of immune organs. However, the exact roles and functional mechanisms need more in-depth studies. In addition, as can be seen from the results, *pRspo2* gene is mainly expressed in the duodenum and ileum, with no expression in the jejunum and rectum.

In summary, we have cloned the ORF of *pRspo2*, analyzed its sequence and predicted the structure and function. Tissue expression analysis of *pRspo2* may facilitate future studies on its detailed functions and potential mechanisms in swine.

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