Rapid Communication: Porcine CRTC3 gene clone, expression pattern, and its regulatory role in intestinal epithelial cells¹

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ABSTRACT: In the current study, we aimed to clone the full-length cDNA of porcine CRTC3 (pCRTC3) gene and examine its expression pattern and function in intestinal epithelial cells. The full-length cDNA sequence of pCRTC3 was 2,173 bp (GenBank accession no. MF964215), with a 1,860-bp open reading frame encoding a 620-AA protein. Comparison of the deduced AA sequence with different species including human, mouse, rat, *Papio*, cattle, and rabbit showed 89% to 91.9% similarity. The pCRTC3 was highly expressed in small intestine and spleen, to a lesser degree in lung, liver, and adipose tissue, and was expressed at a low but detectable level in skeletal muscle, kidney, and heart. In addition, high protein levels of pCRTC3 were found

in IPEC-J2 cells, in which pCRTC3 was mainly localized in cytoplasm. Furthermore, we demonstrated that knockdown of pCRTC3 significantly decreased the expression of the porcine tight junction-related genes including zonula occludens-1 (ZO-1), ZO-2, occludin, and claudin-1 by 57.88% ($P < 0.01$), 40.19% ($P < 0.01$), 51.59% ($P < 0.01$), and 35.70% ($P < 0.05$), respectively. Taken together, we first cloned the full-length sequence of pCRTC3 and revealed the tissue-specific expression pattern, localization, and function of pCRTC3 in regulating the expression of intestinal tight junction-related genes. This study could provide some useful information for understanding the function of CRTC3 in pigs.

Key words: CRTC3, cloning, IPEC-J2, gene expression, pig

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INTRODUCTION

The cAMP–PKA signaling, plays crucial roles in several cellular processes, is mediated through the cAMP-responsive element-binding protein (CREB) and CREB-regulated transcription coactivators (CRTCs). CRTCs bind to CREB and

enhance CREB target genes expression (Iourgenko et al., 2003; Bruno et al., 2014). CRTC3 is one of the CRTCs that involves a variety of biological processes (Song et al., 2010; Liu et al., 2018). It has been demonstrated that CRTC3 is closely associated with obesity and glucose and lipid metabolism (Song et al., 2010; Altarejos and Montminy, 2011; Prats-Puig et al., 2016). Global knockout of CRTC3 enhances energy expenditure and protects the mutant mice against obesity (Song et al., 2010). In white adipocytes, CRTC3 affects glucose uptake and glucose transporter type 4 (GLUT4) expression (Henriksson et al., 2015). In brown adipocytes, the localization of CRTC3 affects uncoupling protein 1 (UCP1) expression (Shan et al., 2016). In skeletal muscle, overexpression

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of CRT3 increased intramuscular triglycerides and regulated myosin heavy-chain protein synthesis (Bruno et al., 2014). These results suggest that CRT3 is a key factor that can regulate glucose and lipid metabolism in adipocytes and skeletal muscle (Liu et al., 2018). As these studies were conducted in mice or human cells, there were no studies have been carried out to characterize porcine CRT3 (pCRT3). Particularly, the function of pCRT3 in intestinal epithelial cells has been completely unknown. Therefore, in the current study, we cloned the full-length of pCRT3 and investigated the tissue-specific expression pattern of pCRT3 in pigs. Furthermore, the localization and function of pCRT3 in porcine IPEC-J2 cells was also studied because high levels of pCRT3 were found in intestinal epithelial cells.

MATERIALS AND METHODS

All procedures were approved by the University of Zhejiang Institutional Animal Care and Use Committee.

Experimental Animals and Sample Collection

In this study, six Duroc × Landrace × Yorkshire pigs at 60 d of age were selected and euthanized. Different tissues including heart (HEA), liver (LIV), spleen (SPL), lung (LUN), kidney (KID), small intestine (INT), longissimus dorsal skeletal muscles, and abdominal adipose tissues (AAT) were collected and rapidly frozen in liquid nitrogen, then stored at −80 °C until analysis.

Cloning of the Full-Length cDNA of pCRT3

To obtain the full-length cDNA sequence of pCRT3, 3'-rapid amplification of cDNA ends technology (RACE) was carried out according to the previous studies (Shan et al., 2009). The primers used for cloning was presented in [Supplementary Table 1](#). The 3'-end of pCRT3 was amplified by nested PCR using a combination of the adaptor primer and gene-specific primer N1 and N2 separately (accession no. XM_005658381.2). The gene-specific primer F1, R1, F2, R2, F3 were designed based on the partial sequence of pCRT3 information database (accession no. XM_021098911.1). R3 was designed based on our 3'-RACE result. The resulting PCR products were cloned, sequenced, and spliced. The full-length porcine CRT3 cDNA was obtained and deposited at GenBank (accession no. MF964215).

Sequence Alignment and Comparison Analysis

After obtaining the full-length cDNA sequence of pCRT3, alignment and comparison analysis were carried out according to the previous studies (Shan et al., 2009). Unrooted neighbor-joining phylogenetic tree was constructed with MegaAlign software package with DNASTAR Lasergene. The accession numbers of CRT3 of other species used for alignment and comparison were downloaded from Genbank.

Cell Culture and Transfection of pCRT3 sgRNA Plasmids

Porcine intestinal columnar epithelial cells IPEC-J2 were cultured in Dulbecco's modified Eagle medium/HamF12 (DMEM/F12, YOCON, Beijing, China) medium containing with 10% fetal bovine serum (GIBCO, New Zealand) and 100 U of penicillin and streptomycin at 37 °C under a humidified atmosphere of 95% air: 5% CO₂. Medium was changed every 2 d. Before transfection, IPEC-J2 cells were seeded on 6-well (35 mm) tissue culture plates (Thermofisher, Carlsbad, CA) at a density of 1.2×10^6 cells/well. The pCRT3-sgRNA1-4 plasmids were transfected into IPEC-J2 using Lipofectamine 2000 (Thermofisher) following the manufacturer's instructions. After 48-h incubation, cells were harvested for examining the expression of related genes.

Immunostaining

Immunostaining was conducted as previously described (Shan et al., 2016). Briefly, IPEC-J2 cells were fixed with 4% PFA and incubated with blocking buffer (5% goat serum, 2% BSA, 0.2% triton X-100, and 0.1% sodium azide in PBS) for 1 h. Then the samples were incubated with CRT3 primary antibody (Abcam, Cambridge, MA) at 4 °C overnight. After washing with PBS, the cells were incubated with second antibody and Hoechst for 45 min at room temperature. Fluorescent image was captured as single-channel grayscale images using a ZEISS Axio Observer3 fluorescent microscope with a ×20 objective (NA 0.70).

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from tissue samples and cultured IPEC-J2 cells using TRIzol reagent (Thermofisher). RNA quality and concentration

were determined using the A260/A280 ratio. About 2 µg of RNA was subjected to reverse transcription with the use of random primer and ReverAid First Strand cDNA Synthesis Kit (ThermoFisher). The quantitative PCR (qPCR) was performed using Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and FastStart Universal SYBR Green Master. Primers used for qPCR were provided in [Supplementary Table 1](#). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression normalized against 18S rRNA as internal control.

Protein Extraction and Western Blot Assay

Total protein was extracted from cultured IPEC-J2 cells using Radio-Immunoprecipitation Assay (RIPA) lysis buffer (50 mmol/mL Tris-HCl, pH 7.4, 150 mmol/mL NaCl, 2 mmol/mL EDTA, 1% SDS, 1% Triton X-100, 10% glycerol, 5 g Na deoxycholate) containing complete protease inhibitor. The protein concentrations were determined using a BCA Protein Assay Reagent Kit (ThermoFisher). Proteins separation and western blot analysis were conducted as previously described ([Shan et al., 2016](#)). The CRTC3 antibody was from Abcam (Abcam), the Occludin and Claudin antibodies were from Proteintech (Proteintech, Hubei, China), and GAPDH antibody was bought from Huabio (Huabio, Zhejiang, China).

Data Analysis

Statistical analysis data are presented as the mean $\pm$ SEM. Comparisons were made by unpaired two-tailed Student's *t*-tests. Effects were considered as significant at $P < 0.05$.

RESULTS

pCRTC3 Full-Length cDNA Clone

In the 3'-RACE experiment, a 521-bp cDNA sequence was amplified. Then we obtained three fragments (445 bp, 693 bp, 716 bp) by PCR reaction using the different pCRTC3-specific primers designed according to pCRTC3 partial sequence (accession No. XM_021098911.1) and our 3'-RACE amplified sequence. By assembling the fragments, a 2,173-bp cDNA of pCRTC3 was obtained, which contained an 1,860 bp ORF that encodes a 620-AA protein ([Supplementary Fig. 1](#)). The 3'-untranslated region contained

a potential polyadenylation consensus signal sequence (AATGAA) downstream of the termination of translation signal TAA ([Supplementary Fig. 1](#)).

Alignment and Phylogenetic Tree Analysis

Comparison of the full-length coding sequences of pCRTC3 with the human, mouse, rat, *Papio*, horse, monkey, dog, cat, and rabbit CRTC3 showed 89.2%, 86.6%, 87.7%, 89.7%, 88.9%, 88.8%, 82.7%, 84.7%, and 87.7% similarity, respectively (data not shown). BLAST analysis of the AA sequence of pCRTC3 with those of the other species indicated that pCRTC3 shared 90.6% similarity with human, 89% with mouse, 89.8% with rat, 91.9% with *Papio*, 91.3% with cattle, and 89.2% with rabbit ([Fig. 1A](#)). The alignment and phylogenetic tree analysis results revealed that pCRTC3 is more closely linked to cattle than fish and *Xenopus* ([Fig. 1B](#)).

Expression Pattern of pCRTC3

Tissue-specific expression pattern of pCRTC3 was determined by western blot and qPCR. Western blot results showed that pCRTC3 protein levels were highly expressed in SPL and small INT, to a lesser degree in LIV, LUN, and adipose tissue, and was expressed at a low but detectable level in KID, skeletal muscle and HEA ([Fig. 2A](#)). Results from qPCR further confirmed that pCRTC3 was highly expressed in small INT and SPL ([Fig. 2B](#)). In addition, high protein levels of pCRTC3 were found in IPEC-J2 cells ([Fig. 2C](#)). Moreover, immunostaining results indicated that pCRTC3 is mainly localized in the cytoplasm under basal conditions ([Fig. 2D](#)).

pCRTC3 Regulates the Expression of Epithelial Tight Junction-Related Genes

As high levels of pCRTC3 were found in small INT and IPEC-J2 cells, the function of pCRTC3 in intestinal epithelial cells has been examined. Western blot results demonstrated that transfection of pCRTC3-sgRNA dramatically decreased the protein expression of pCRTC3 in IPEC-J2 cells compared to the control groups that only transfect with Lipofectamine 2000 or pX330-Cas9 empty vectors ([Fig. 2E](#)). Notably, the knockdown efficiency of pCRTC3-sgRNA1 and pCRTC3-sgRNA4 were better than pCRTC3-sgRNA2 and pCRTC3-sgRNA3 ([Fig. 2E](#)). Furthermore, qPCR results shown

Figure 1. Alignment and phylogenetic tree analysis results of the CRTC3 family proteins. (A) Amino acid alignment of CRTC3 of different species. (B) The phylogenetic tree showing the evolutionary relationship between different species.

that transfection of pCRTC3-sgRNA1 significantly decreased the pCRTC3 mRNA level by 52.01% ($P < 0.01$) compared to the pX330-Cas9 empty vector control group. Interestingly, pCRTC3-sgRNA1 treatments significantly reduced the mRNA levels of zonula occludens (ZO)-1, ZO-2, occludin, claudin-1, and claudin-2 by 57.88% ($P < 0.01$), 40.19% ($P < 0.01$), 51.59% ($P < 0.01$), and 35.70% ($P < 0.05$), respectively, compared to the pX330-Cas9 empty vector control groups (Fig. 2F).

DISCUSSION

In the current study, we first cloned the full-length cDNA sequence of pCRTC3 and determined the expression pattern of pCRTC3 in different tissues. Furthermore, we investigated the regulatory role of pCRTC3 in epithelial cells. We demonstrated that pCRTC3 was highly expressed in small INT and intestinal epithelial IPEC-J2 cells. Moreover, knockdown of pCRTC3 using pCRTC3-sgRNA significantly decreased the expression on tight junction-related genes in IPEC-J2 cells. These

Figure 2. Expression pattern and regulatory role of pCRTC3 in intestinal epithelial cells. (A) The protein levels of pCRTC3 in heart (HEA), liver (LIV), spleen (SPL), lung (LUN), kidney (KID), intestine (INT), dorsal skeletal muscles (DMU), and abdominal adipose tissues (AAT) of 65-d-old pigs. (B) The relative mRNA abundance of pCRTC3 in various tissues ($n = 6$). The expression of pCRTC3 in other tissues was normalized to that in AAT. (C) The protein levels of pCRTC3 in small intestine tissue (INT) and IPEC-J2 cell (IPE). (D) Localization of pCRTC3 in IPEC-J2 cells determined by immunostaining. (E) Western blot results of IPEC-J2 cells treated with pCRTC3-sgRNA1-4 and Lipofectamine 2000; only Lipofectamine 2000; Cas9, Lipofectamine 2000 + pX330-Cas9 empty vector; sg1-4, Lipofectamine 2000 + pCRTC3-sgRNA1-4. (F) The mRNA levels of CRTC3 and tight junction-related genes in control pX330-Cas9 empty vector and pCRTC3-sg-RNA1 transfected IPEC-J2 cells. Each column represents the means $\pm$ SEM ($n = 3$). * $P < 0.05$; ** $P < 0.01$. Scale bar, 50 μ m.

results demonstrated that pCRTC3 could regulate the expression of intestinal tight junction protein genes and may play regulatory role in regulating the development and intestinal barrier of intestine.

CRTC3 is a coactivator of CREB and plays crucial roles in regulating lipid and energy metabolism (Song et al., 2010; Liu et al., 2018). More recently, Lee et al. (2018) performed a genomic characterization of pCRTC3 and shown that p.V515F mutation in the pCRTC3 affects lean meat production and belly fat. Given the important roles of CRTC3 and limited studies on pCRTC3, we cloned the full-length sequence of pCRTC3. The full-length cDNA sequence of pCRTC3, which encodes a 620-AA protein, was cloned. Likewise, human CRTC3 protein has an open reading frame of 1,857 bp, which yields a 619-AA protein (Iourgenko et al., 2003), whereas the mouse CRTC3 encodes a 619-AA protein (Song et al., 2010). The CRTC3 family

have similar structures with evolutionary conservatism (Iourgenko et al., 2003; Liu et al., 2018). Here, we found that the AA sequence of pCRTC3 shared about 89–91.9% similarity with the AA sequences of CRTC3 in other species we compared. Phylogenetic tree analysis results shown that pCRTC3 is more closely linked to mammals such as cattle than fish and *Xenopus*, suggesting CRTC3 is a highly conserved protein.

CRTC3 is widely expressed in a variety of tissues, especially in adipose tissue in mice (Song et al., 2010; Altarejos and Montminy, 2011) and in LUN in human (Wu et al., 2006). Consistent to the previous study (Song et al., 2010), we also found that pCRTC3 is highly expressed in LUN and adipose tissue. Likewise, Lee et al. (2018) reported that backfat tissues expressed higher mRNA levels of CRTC3 than longissimus muscle. Moreover, we found that the mRNA and protein levels of pCRTC3 in small INT

were higher than that in LUN and adipose tissue. Likewise, comparable mRNA levels of CRTC3 were found in human small INT (Wu et al., 2006). In addition, high protein levels of pCRTC3 were found in intestinal epithelial IPEC-J2 cells, in which pCRTC3 is mainly localized in cytoplasm. Consistent with our results, CRTC3 was mainly localized in cytoplasm of brown adipocytes in mice (Shan et al., 2016). Taken together, we demonstrated that pCRTC3 is highly expressed in intestinal epithelial cells.

Porcine IPEC-J2 intestinal epithelial cells, a widely used intestinal cell line, is derived from neonatal piglet mid-jejunum (Brosnahan and Brown, 2012; Zhou et al., 2017). The polarization of IPEC-J2 cells express tight junction proteins (e.g., claudins3 and occludin) and form tight junctions (Schierack et al., 2006). Tight junctions are the basic structure of intestinal barrier, which serve as the rate-limiting barrier to passive movement across intestinal epithelial cell (Garcia-Hernandez et al., 2017). Here, we found that knockdown of pCRTC3 decreases the expression of tight junction-related genes including ZO-1, ZO-2, occludin, claudin-1, and claudin-2. The expression of tight junctional protein significantly decreased in pCRTC3-sgRNA1 group demonstrated that pCRTC3 may affect tight junction function of intestine. As previously reported, overexpression of CRTC3s disrupted the tight junction protein Bbg in flies and caused constitutive upregulation of innate immunity genes of immune response that maintains epithelial integrity and gut homeostasis (Shen et al., 2016). Other group reported that CRTC3s acting as a conserved substrate of Ca^{2+} -dependent phosphatase calcineurin promotes intestinal somatic stem cells proliferation (Deng et al., 2015). Together, these results suggested that CRTC3 may play an important role in intestine. However, more studies are needed to clarify the exact role of pCRTC3 in the development and barrier formation of porcine intestine.

In conclusion, we first cloned the full length of pCRTC3 and examined the tissue-specific expression pattern of pCRTC3 in different tissues of pigs. Furthermore, we demonstrated that pCRTC3 is mainly localized in cytoplasm and could regulate tight junction-related genes expression in IPEC-J2 cells. These results could provide new insights into understating the function of pCRTC3 in pigs.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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