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Molecular characterization, tissue expression profile, and single nucleotide polymorphism analysis of the *periostin* gene in swine

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ABSTRACT. Periostin, also called osteoblast-specific factor 2, is an important regulator of bone, cardiac development, and wound healing. A recent study revealed that *periostin* plays an important role in tumor development and is upregulated in a wide variety of cancers. However, little is known about *periostin* in swine. Therefore, the cDNA sequence of the porcine *periostin* gene was obtained by rapid amplification of cDNA ends (RACE). One *C*/*T* single nucleotide polymorphism anchored in intron 9 was identified and genotyped by PCR-RFLP-*Hae*III. In Daweizi, Shaziling, Ningxiang, Taoyuan, Wuzhishan, Landrace, and Yorkshire pigs, the *C* allele was dominant, while the *T* allele was dominant in the Duroc pig. Quantitative PCR analysis showed that the *periostin* gene was expressed in all examined tissues from 25-day-old Shaziling and Yorkshire piglets, with mRNA expression in the longissimus dorsi muscle being the highest in these two breeds, and that in the kidney and lungs being the lowest. There was a significant difference in *periostin* gene expression in the

intestines, heart, and spleen ($P < 0.05$). These findings might contribute to our understanding of the function of periostin in swine.

Key words: Pig; *Periostin*; Expression profile; SNP

INTRODUCTION

Periostin is an important extracellular matrix (ECM) protein belonging to the fasciclin family of proteins (Wang and Mao, 2007), which promotes the proliferation and migration of cells (Tsunoda et al., 2009). In the long-term overload of heart stimulation and myocardial infarction, *periostin* plays a key role in the development of cardiac hypertrophy, interstitial fibrosis, and ventricular remodeling (Norris et al., 2004), and is also involved in wound healing (Roy et al., 2007). Periostin is an important regulatory factor involved in the development and remodeling of bone, and following a fracture, the expression of *periostin* mRNA was found to increase 2-fold, and was associated with osteogenic precursor cells in periosteal and undifferentiated mesenchymal cells near the fracture site (Nakazawa et al., 2004). Periostin is also involved in the growth of endometrial blood vessels and the development of atherosclerosis (Bagnato et al., 2007). In addition, a relationship between the *periostin* gene and the presence and development of atherosclerosis has been found. In the rat carotid artery balloon injury model, the level of *periostin* mRNA and protein expression significantly increased in the damaged artery intima (Lindner et al., 2005). Furthermore, experiments have shown that *periostin* mRNA expression increased significantly in rat pulmonary artery smooth muscle cells under a hypoxic stress response, which is similar to the process that occurs during atherosclerosis (Li et al., 2004). The results of other studies have shown that *periostin* can regulate breast cancer cells and the expression of kinase domain receptor in human capillary endothelial cells (Försti et al., 2007; Binion et al., 2008). Transforming growth factor-beta 1 (TGF-β1) was found to induce periostin production, and this finding has been confirmed in colon cancer cells (Tai et al., 2005). In addition to TGF-β1, bone morphogenetic protein, platelet-derived growth factor (PDGF-aa, PDGF-bb), and fibroblast growth factor (FGF-B, FGF-A) were potential factors for star-shaped cells secreted periostin (Erkan et al., 2007). Fluorescence *in situ* hybridization has shown the *periostin* gene to be located on *HSA*13q13.3 in humans and on mouse chromosome 8A1.1-1.2 (Takeshita et al., 1993). Integrin-associated periostin can activate Akt/protein kinase B- and focal adhesion kinase-mediated signaling pathways, which increase cell survival and angiogenesis, invasion, metastasis, and importantly, epithelial to mesenchymal transition of carcinoma cells (Morra and Moch, 2011). The *periostin* gene is expressed abundantly in human and mouse, but no study has investigated its effect on meat quality in pigs. In this study, the cDNA sequence of the *periostin* gene was cloned by rapid amplification of cDNA ends (RACE) using tissue samples from pigs, and expression profiling of *periostin* mRNA in different tissues from different breeds was studied using quantitative PCR (q-PCR). This investigation aimed to lay a foundation for the further study of periostin in swine.

MATERIAL AND METHODS

Generation of cDNA

Total RNA was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) from the longissimus dorsi muscle of 25-day-old Yorkshire pigs (Zhang et al., 2010). RNA was treated with

DNase I (Liang and Pardee, 1992) and then reverse-transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA). The human *periostin* gene sequence (NM_001286667) was applied and compared with all sequences in the expressed sequence tags (EST) database using a standard BLAST (http://www.ncbi.nlm.nih/gov/blast/), and the porcine ESTs that shared at least 80% identity to the corresponding human mRNA were selected and used to design gene specific primers (Table 1). The PCR products were purified with the 3S Spin DNA Agarose Gel Purification system (Shenergy Biocolor, Shanghai, China) and cloned into the pMD18-T vector (TakaRa, Dalian, China), then sequenced by Shanghai Biosune Co. Ltd. (Shanghai, China).

Table 1. Sequences of primers used to perform PCR amplifications.

Primers purpose	Primer name	Primer sequence (5'-3')	Tm (°C)	Product size (bp)
Cloning	RC11-1F	GAAGAACCCAGATTTTGTCAGC	55	817
	RC11-1R	CTCTACTTCCCAAAAGGTCTCC		
	RC11-2F	CAGTGCTTCTCCCTGACCGA	55	733
	RC11-2R	CTTTATTTTCCCATAGTTTTAGGTCAGA		
Polymorphism	Genomic-F	AGGAGCACTTATTCTTGT	59	1249
	Genomic-R	AAATGCGTTATTCACAGG		
Expression profile	Periostin-F	AGCAAACCACTTTCACAGACCT	58	101
	Periostin-R	AAATGCGTTATTCACAGGCG		
Internal control	*GAPDH*-F	ATTTGGCTACAGCAACAGGGT	59	172
	GAPDH-R	AAGTCAGGAGATGCTCGGTGT		

SNP identification and PCR-RFLP test

Blood samples were collected from the ears of eight swine breeds, including Duroc pigs (N = 57), Landrace pigs (N = 38), Yorkshire pigs (N = 360), Shaziling pigs (N = 63), Daweizi pigs (N = 62), Taoyuan pigs (N = 70), Ningxiang pigs (N = 77), and Wuzhishan pigs (N = 55), and genomic DNAs were amplified and sequenced directly for the identification of single nucleotide polymorphisms (SNPs). The PCR for genotyping was performed in a volume of 20 μL consisting of 100 ng genomic DNA, 10X buffer, 0.5 μM each primer, 75 μM each dNTP, 1.5 mM $MgCl_2$, and 0.5 U *Taq* DNA polymerase (Promega). The PCR protocols were 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50.5°C, 1 min at 72°C, and a final extension of 10 min at 72°C. A total of 4 μL PCR product was digested overnight with 3 U *Hae*III (Thermo Fisher Scientific, USA) at 37°C, then size-separated on 2.0% agarose gel stained with ethidium bromide.

Expression profile of *periostin* gene

Total RNA was isolated from 10 tissues (heart, liver, spleen, lung, kidney, intestine, cecum, pancreas, crureus, and longissimus dorsi muscle) of a 25-day-old weaned Yorkshire piglet and a Shaziling piglet of the same age (Winer et al., 1999; Zhang et al., 2010). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. The expression profile and internal control primers were designed according to the pig mRNA sequence. Analysis of relative gene expression was conducted using real-time qPCR and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

RESULTS

cDNA sequence

Cloning and characterization of the porcine *periostin* cDNA sequence was performed using the RACE method, and the full-length cDNA was 2952 bp. It contained an open reading frame (ORF) of 2346 bp, a 5'-untranslated region (UTR) of 118 bp, and a 3'- UTR of 488 bp, encoding 781 amino acids. This gene was deposited to GenBank under the accession No. KF500034.

Periostin cDNAs from 12 species obtained from the NCBI database were used for phylogenetic tree analysis, including *Pan troglodytes* (XM_001148381.3), *Mus musculus* (NM_015784.3), *Oryzias latipes* (NM_001305404.1), *Rattus norvegicus* (NM_001108550.1), *Canis lupus familiaris* (LC019996), *Homo sapiens* (NM_006475.2), *Bos taurus* (NM_001040479.1), *Aotus nancymaae* (XM_012469826.1), *Aquila chrysaetos canadensis* (XM_011580807.1), *Ovis aries musimon* (XM_012130755.1), and *Macaca nemestrina* (XM_011748726.1). The results showed that the *periostin* cDNA sequences from different animals formed one subgroup. The highest homology was found between sheep and cattle sequences, and the lowest homology was with *Oryzias latipes* (Figure 1).

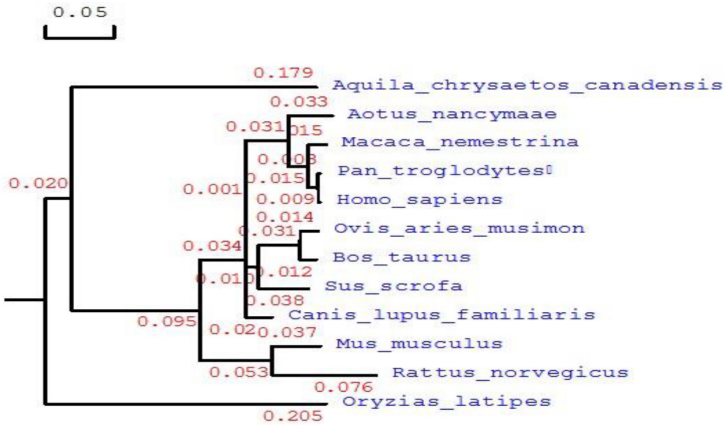

Figure 1. Phylogenetic tree of the cDNA sequences of periostin. The tree was constructed using the multiple-sequence alignment method in the DNAMAN 6.0 software.

Polymorphism detection

One SNP anchored in intron 9 of the *periostin* gene was identified. A genomic fragment of 1249 bp was amplified and subsequently genotyped with PCR-RFLP-*Hae*III (1249 bp for allele *T*, and 708 and 541 bp for allele *C*) (Figure 2).

The gene and genotype frequencies were calculated for the eight pig breeds and independence tests of the *periostin* gene were conducted (Table 2). The chi-square test showed that there were significant differences between genotypes in the different breeds ($P < 0.05$). In Taoyuan and Wuzhishan pigs, the genotype frequencies were not significantly different ($P > 0.05$). Phenotype values of pigs carrying the *TT* genotype were significantly lower than those carrying the *CC* genotype ($P < 0.05$; Table 2). Allele *C* was predominant in these pig breeds, except for in Duroc pig (Table 2), the highest *C* gene frequency was 0.9921 in the native breed, Shaziling pig, the *T* gene frequency was 0.7097 in the European breed Duroc pig, and the *T* allele was not found in

the Taoyuan and Wuzhishan pig breeds. From the genotype frequency distribution, *CC* was the dominant genotype.

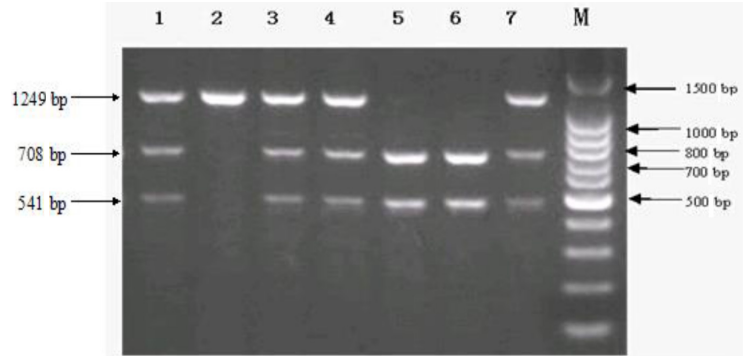

Figure 2. One single nucleotide polymorphism (SNP) was detected in the *periostin* gene. Polymerase chain reaction (PCR) products were digested with *Hae*III to distinguish different alleles. Agarose gel (2%): *lanes 1-7*: *TT* genotype: *lane 2*, *CC* genotype: *lanes 5* and *6*, *CT* genotype: *lanes 1*, *3*, *4*, and *7*. *Lane M* = DL100-bp DNA ladder.

Table 2. Frequency of alleles and genotypes of the *periostin* gene determined by PCR-RFLP-*Hae*III among different pig breeds.

Breed	Sample size	Genotype			Frequency of genotype			Frequency of allele		χ^2
		TT	*CT*	*CC*	*TT*	*CT*	*CC*	*T*	*C*	
Ningxiang	77	0	31	46	0	0.5974	0.4026	0.2013	0.7987	4.8911
Daweizi	62	0	3	59	0	0.0484	0.9516	0.0242	0.9758	0.0381
Shaziling	63	0	1	62	0	0.0159	0.9841	0.0079	0.9921	0.0040
Taoyuan	70	0	0	70	0	0	1	0	1	-
Wuzhishan	55	0	0	55	0	0	1	0	1	-
Yorkshire	360	44	145	171	0.1222	0.4028	0.475	0.3236	0.6764	2.3006
Landrace	38	0	21	17	0	0.5526	0.4474	0.2763	0.7237	5.5398
Duroc	62	35	18	9	0.5645	0.2903	0.1452	0.7097	0.2903	5.4122

Expression profile analysis

RT-PCR was used to investigate the levels of *periostin* mRNA from the total RNA extracted from different tissues (heart, liver, spleen, lung, kidney, intestine, cecum, pancreas, crureus, and longissimus dorsi muscle). Quantitative PCR showed that the porcine *periostin* gene is broadly expressed in the 10 tissues examined from Yorkshire and Shaziling pigs (Figure 3). The housekeeping gene *GAPDH* was used as an endogenous reference for the determination of target mRNA expression, and the expression calibrator was set as 10 in spleen tissue from Shaziling pig for subsequent relative quantification of periostin mRNA levels. *Periostin* mRNA showed the highest expression in the longissimus dorsi muscle and almost no expression was found in the kidney and lungs from these two breeds ($P < 0.01$). The expression in the heart and spleen of Yorkshire pigs was higher than that in Shaziling pig ($P < 0.05$), and lower in the intestine ($P < 0.05$).

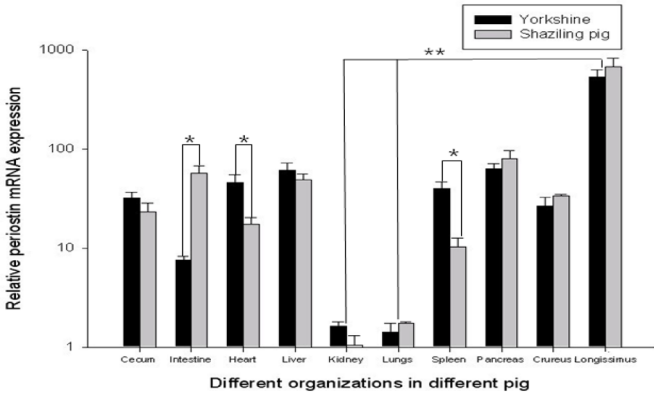

Figure 3. Comparison of the expression profiles of *periostin* mRNA in different tissues from 25-day-old Yorkshire and Shaziling pigs. *Indicates P < 0.05, **Indicates P < 0.01.

DISCUSSION

Periostin, which was originally identified in a mouse (*M. musculus*) osteoblast cell line, has a molecular weight of 90 kDa, consists of 22 exons, 21 introns, and 811 amino acids, and is an ECM protein that is secreted by cardiac fibroblasts. The mouse *periostin* gene was located on chromosome 3.3C (Takeshita et al., 1993). The human (*H. sapiens*) *periostin* gene was located on chromosome 13q13.3, and contains 22 exons and 21 introns, encodes 761 amino acids, and is an important candidate gene for cardiovascular disease. Periostin contains an N-terminal signal peptide, a cysteine-rich region, four internal homologous repeat sequences, and a C-terminal hydrophilic zone. Periostin is secreted by stromal-specific cells, and is mainly distributed in the ECM where it directly interacts with a variety of ECM proteins such as collagen I and IV, and fibronectin, to maintain the stability of the ECM environment (Snider et al., 2008). The N-terminal signal peptide sequence and four internal homologous repeat sequences mediate the association of periostin with integrins in the cell membrane, which mediate cell-to-cell and ECM adhesion (Litvin et al., 2005). Periostin also plays an important role in the repair of ECM (Suzuki et al., 2004), formation of the cytoskeleton, differentiation of fibroblasts (Snider et al., 2008), and the pathological and physiological processes of bone matrix remodeling. Although we found that the *periostin* gene was located on chromosome 11 in pigs (Ma et al., 2005), no further studies investigating the *periostin* gene in pigs have been reported. In this study, we performed RACE-PCR and finally obtained a 2952-bp full-length cDNA sequence, with a 2346-bp ORF flanked by a 118-bp 5'-UTR and a 488-bp 3'-UTR, encoding 781 amino acids. The cDNA sequences shared 92 and 86% identity with the human (GenBank accession No. NM_001286667) and mouse (GenBank accession No. NM_015784.3) gene sequences, respectively, and the encoded amino acid sequences shared 89 and 88% identify, respectively, indicating that the *periostin* gene is highly conserved in mammals.

Our results indicate that allele *C* was dominant in all pig breeds studied except for Duroc pigs. The *TT* allele was not detected in the native pig breeds or in Landrace pigs, and the *T* allele was not detected in the Taoyuan and Wuzhishan pigs. This result could be due to long-term directional selection, and the sample size should be expanded for further study. The chi-square test showed that genotypes differed significantly between different breeds (P < 0.05). In Taoyuan and Wuzhishan pigs, the genotype frequencies showed no significant difference (P > 0.05); however,

highly significant differences exist between these two breeds and Duroc pigs ($P < 0.05$). The differences of the interaction were not significant in the remaining species studied ($P > 0.05$). The site was located in the conserved region of *periostin* genes, it is likely that the mutation observed within the conserved region had an impact on gene function. Therefore, the differences in *periostin* genotype frequencies in different varieties suggest that the site C12835T may have an impact on related traits.

In this study, C12835T on intron 9 of the *periostin* gene did not cause an amino acid change, but it could regulate the expression of amino acids in downstream exons. Whether the mutation affected mRNA expression, growth, or development remains to be determined. In living cells, the transcription of functional genes and protein translation ensures homeostasis of biochemical reactions, and overexpression of proteins could enable the transcription and translation of upstream mRNA encoding genes as part of negative feedback regulation. Periostin protein function in organisms, the expression levels of *periostin* mRNA and protein levels in relationships, and mutual adjustment mechanisms remain to be studied. To gain a better understanding of the role of *periostin* in pigs before the early stages of the growth and development, we examined the mRNA expression of *periostin* in 10 tissues from Shaziling and Yorkshire piglets. Differences in *periostin* mRNA expression between the two pig breeds showed that expression in longissimus dorsi muscle tissues was higher than in other tissues, and almost no expression was observed in the kidney and lungs. Muscle tissue is composed of muscle fibers, and, according to the oxidation characteristics, muscle fibers can be classified into slow-oxidative (type I), fast-oxidative (type IIa), fast-glycolytic (type IIb), and intermediate (type IIx) fibers (Bottinelli and Reggiani, 2000). Early growth stages in animals are important for the metabolism of muscle fiber and conversion of contraction type (Yang et al., 2008). At 3-20 days old, the proportions of MyHC type I, type IIa, type IIx, and type IIb fibers were significantly changed in longissimus dorsi muscle, in which type I, type IIa, and type IIx fibers were significantly reduced, while type IIb fibers were significantly increased (Xiaojing et al., 2005). The high expression of *periostin* in the longissimus dorsi muscle may be associated with the formation of muscle fibers in pig. Highly significant differences in expression between the kidney, lung, and longissimus dorsi muscle tissues were found by the chi-square test ($P < 0.01$), and there was no significant difference between the expression in the rest of the tissues ($P > 0.05$). *Periostin* mRNA was highly expressed in the heart, intestine, and spleen ($P < 0.05$). The mRNA levels of *periostin* in crureus and longissimus dorsi muscle tissues from Shaziling piglets were higher than from those in Yorkshire piglets at 25 days old, which may be related to its fine meat quality.

In summary, the full-length cDNA of the pig *periostin* gene was isolated and characterized, and one *C/T* SNP, which relates to meat quality traits, was identified in intron 9 of the *periostin* gene. The spatial and temporal expression profiles of pig *periostin* mRNA showed abundant expression in longissimus dorsi muscle tissue, and different expression patterns were observed between two breeds with genetically distinctive backgrounds.

Conflicts of interest

The authors declare no conflict of interest.

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