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Molecular characterization, expression patterns, and promoter activity analysis of *PGM1* in pigs

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ABSTRACT. The phosphoglucomutase 1 (*PGM1*) gene was differentially expressed in tissues of Chinese Meishan and Large White pigs. In this study, the promoter region, expression profile, and genetic mutations of the gene were determined. Expression of a 5'-deletion in both C2C12 and PK-15 cells showed that a negative regulatory element was at -1871 to +185 bp and a positive regulatory element was at -1158 to +185 bp. Among the different types of muscle fibers, *PGM1* had the highest expression in both longissimus dorsi and biceps femoris. The expression was concentrated in the muscle fibers at different growth stages of Meishan and Large White pigs. The synonymous mutation C462T in the coding sequence was confirmed by polymerase chain reaction-restriction fragment length polymorphism, and the frequency of the C allele was dominant in Chinese indigenous breeds. Association analysis with lean meat showed that the C462T site was different.

Key words: *PGM1*; Gene function; Skeletal muscle; Pig; Promoter region

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INTRODUCTION

Chinese indigenous pig breeds such as Taihu, Tongcheng, and Meishan often have valuable traits such as disease resistance, high fertility, good maternal qualities, unique product qualities, longevity, and adaptation to harsh conditions. Western pig breeds such as Large White, Landrace, and Duroc show good achievements in growth rate and high lean rate; in particular, these introduced pigs have a high lean meat rate and food conversion efficiency, whereas Chinese indigenous pigs have more fat deposition and superior meat quality (Li et al., 2003). Phenotypic variances were mainly determined by the genetic differences. Therefore, detecting the genetic differences between Chinese indigenous and Western pig breeds or determining the differentially expressed genes between Chinese indigenous and Western pig breeds that determine these phenotypic variances is important to pig breeders (Wang et al., 2012).

The number and size of myofibers are critical for meat production and quality (Rehfeldt et al., 2000). In the pig, the total number of muscle fibers is determined at the prenatal stages, while the fiber size is determined in the postnatal development process (Dwyer et al., 1993). There are 2 major waves of fiber generation: a primary generation from 35 to about 60 days post-coitus (dpc) and a secondary generation from about 54 to 90 dpc (Ashmore et al., 1973; Wigmore and Stickland, 1983). Differentially expressed genes in the early stages of muscle development may be potential candidate genes to improve meat quality and quantity (Rehfeldt et al., 1999). Improved growth rate and lean meat percentage have been recognized for many years as important objectives for increasing the proficiency and productivity of pig breeding (Wu et al., 2011a).

Phosphoglucomutase 1 (*PGM1*) is a member of the PGM family, which contains *PGM1* to *PGM5* (Insley et al., 1968; Billardon et al., 1973; Edwards et al., 1995). The PGM activity center is a 5-peptide motif including a serine phosphate combination; therefore, it belongs to the class of serine enzymes (Quick et al., 1972). The human *PGM1* gene was cloned in 1993 (Whitehouse et al., 1998). The human *PGM1* gene was localized to human chromosome 1 in 1999, and mutations in this gene were analyzed by fluorescence-based polymerase chain reaction (PCR) for single-strand conformation polymorphism (Looft et al., 1999). The researchers used pathway-focused oligo microarray studies to examine the expression changes of 140 genes associated with meat quality. Clustering analysis revealed that some genes, including porcine *PGM1*, influenced porcine carcass characteristics and meat quality (Xu et al., 2012). There was little information available in the literature about the relationship between *PGM1* and economically important porcine traits. *PGM1* belongs to a group of glucose biosynthesis enzymes, and the function of this gene is closely associated with cell growth regulation and fatty acid biosynthesis (Li et al., 2008).

Therefore, we selected the *PGM1* gene as a potential candidate gene for meat quality, identified the mutations and promoter, and then determined their roles on muscle fiber formation in 2 different pigs.

MATERIAL AND METHODS

Animals and tissues

All pigs were fed at the Jingpin Pig Station of Huazhong Agriculture University (Wuhan, China), and all animal procedures were performed according to protocols approved by

Hubei Province, PR China, for Biological Studies Animal Care and Use Committee.

All samples from embryonic periods and post-periods from Large White and Chinese Meishan pigs were immediately frozen in liquid nitrogen and stored at -80°C.

For expression analysis in different muscles, fast twitch and oxidative samples from the masseter and soleus were collected from three 120-day-old Large White pigs. A female indigenous 150-day-old Meishan pig adult heart, liver, spleen, lung, kidney, stomach, muscle, fat, ovary, uterus, and brain samples were collected; embryonic heart, liver, spleen, lung, kidney, stomach, muscle, and brain samples were also collected for spatial expression analysis.

RNA source, extraction, and cDNA preparation

Total RNA was isolated from frozen tissue (heart, liver, spleen, lung, kidney, stomach, fat, masseter muscle, small intestine, testicle, hippocampi, and pallium) of pig using Trizol reagent (Invitrogen, USA). To further verify that no genomic DNA was present, each sample was treated with RNase-free DNaseI (Promega, USA) to remove contaminating genomic DNA. The integrity of RNA samples was analyzed by ethidium bromide staining of the ribosomal RNAs on 1% formaldehyde denaturing agarose gels, and the quality was assessed by NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), with an optimal 260/280 ratio between 1.8 and 2.0. cDNA was synthesized from 1 µg total RNA in 20-µL reactions using a RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, Canada). After synthesis, the cDNA was diluted 5-10 times with double-distilled water and stored at -20°C.

Plasmid construction, cell culture, transient transfection, and luciferase assay

Six different *PGM1* promoter fragments were amplified by PCR from the genomic DNA using different pairs of primers (P-1, P-2, P-3, P-4, P-5, and P-6 as shown in Table 1) designed according to the *Sus scrofa PGM1* gene sequence from the National Center for Biotechnology Information database. The purified PCR products were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced commercially (Sangon, China). After being confirmed by DNA sequencing, the PCR products were then excised with *Mlu*I and *Xho*I (Fermentas, Japan) and ligated into the pGL3-Basic vector named pGL3(1-6) separately (Promega). All above plasmids were confirmed by restriction enzyme digestion and sequencing.

The pig kidney cells (PK-15) and C2C12 myoblast cells obtained from China Center for Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10% (v/v) bovine calf serum (Gibco), on cell plates under humidified air containing 5% CO_2 at 37°C. In order to analyze the promoter activity in various conditions, cells were seeded at a density of 1.5 x 10^5 cells/mL on 24-well plates using DMEM supplemented with 10% fetal bovine serum (FBS). After 18-24 h, the plated cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer recommendations (plasmid:Lipofectamine 2000 = 1:2.5). Plasmid DNA of every well used in the transfection contained 0.8 ng *PGM1* promoter constructs and 0.04 ng internal control vector PRL-TK *Renilla* luciferase plasmid. After 6 h, the medium was replaced with fresh growth medium containing 10% FBS.

Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')	Size (bp)	Tm (°C)
PGM1 - real-time RT-PCR			
PM1a-F	AGCATTCCGTATTTCCAGCAA	145	59
PM1a-R	TCAGATTCCCAAAAAACTTCCAA		
PGM1 - sequence amplification and SNP detection			
P-1F	CGACGCGTTGTACTGGAAGGTGAGGT	2311	60
P-2F	CGACGCGTAAAGACAGTGAGGAACAG	2056	
P-3F	CGACGCGTACATCCACATAATGCCTC	1633	
P-4F	CGACGCGTGTAGTTTCAAAAGTGTCT	1343	
P-5F	CGACGCGTCTAACCTACAAACACCGT	371	
P-6F	CGACGCGTATAGTGCCAGCCCAGGAA	100	
P-R	CCGCTCGAGCTTCCACTCACGACTATT		
001S-1F-SNP	TGGTCGCTTGGTTATTGG	1742	56
001A-1R-SNP	TGGGTGCTAAGGAGACAAAT		
01E-1F-SNP	GAGGCGTTGCGGATTTCT	913	60
01E-1R-SNP	CTGCTCGGTAACTGTATCTTCG		
11E-1F-SNP	TTGGGATGTCGGAGTAGA	1087	55
11E-1R-SNP	AAGGCAGTGTTGTGCTATTT		
03E-02F-RFLP	GGGCTCTTTGTTTCCTTTTG	427	58
03E-02R-RFLP	CAAACCCCTCAAGCTGGAAC		
03E-01F-RFLP	TGATGTTTGGCTGCCTGAG	759	58
03E-01R-RFLP	TGAGTTTGCGGGTTCGTAT		
β-actin			
β-actin-F	CCAGGTCATCACCATCGG	158	58
β-actin-R	CCGTGTTGGCGTAGAGGT		

Real-time reverse transcription (RT)-PCR analysis

Real-time PCR was performed on a Lightcycler 480 (Roche, France). All reactions were performed in triplicate in a total volume of 20 µL using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Each 20-µL real-time RT-PCR included 10 µL SYBR® Green qPCR SuperMix-UDG, 1 µL cDNA, 0.5 µL primers. PCR conditions consisted of 1 cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 20 s, 59°C for 20 s, and 72°C for 20 s, with fluorescence acquisition at 72°C. Using the Gene Expression Macro software (Bio-Rad, Richmond, CA, USA), we employed an optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method (Livak and Schmittgen, 2001) for analysis. The $2^{-\Delta\Delta Ct}$ equation is valid only if the amplification is equal. All PCR amplifications were performed in triplicate for each RNA sample.

Association analysis of the porcine *PGM1* gene

Association analysis was performed in our experimental populations that contained 259 Meishan x Yorkshire F_2 pigs. A general linear model program of the SAS version 8.0 software package (SAS Institute) was used to evaluate the associations between genotypes and fat traits (Liu, 1997). The model of SAS program was as follows:

$$Y_{ijklmn} = \mu + G_i + S_j + B_k + P_l + W_m + A_n + e_{ijklmn} \qquad \text{(Equation 1)}$$

where μ is the population mean, Y_{ijklmn} is the phenotypic value of the target trait, G_i is the genotype effect, S_j is the sex effect, B_k is the boar effect, P_l is the population stratification effect, W_m is the regression coefficient of the slaughter weight, A_n is the regression coefficient of the slaughter age, and e_{ijklmn} is the random error effect for each observation.

RESULTS AND DISCUSSION

Features of the 5'-flanking region of porcine *PGM1*

A 2311-bp contig in the 5'-flanking region was obtained by PCR. Prediction analysis using the Neural Network Promoter Prediction version 2.2 software program with a score cutoff of 0.8 (http://www.fruitfly.org/seq_tools/promoter.html) and Transcription Factor Binding Site Prediction (http://www.cbrc.jp/htbin/nph-tfsearch) revealed 6 potential transcription promoters at -2126 to +185 bp, -1871 to +185 bp, -1448 to +185 bp, -1158 to +185 bp, -186 to +185 bp, and 85 to +185 bp when the translational start site (ATG) was designated as +1. To determine the location of the promoter activity of the pig *PGM1* gene, we studied the transcriptional activity of recombinants of progressive 5'-deleted DNA fragments (-2126/+185, -1871/+185, -1448/+185, -1158/+185, -186/+185, and +85/+185) linked to the pGL3 reporter (Figure 1). The recombinants were transiently transfected into PK-15 and C2C12 cells. The transcriptional activity was normalized to that of *Renilla* luciferase and then pGL3-Basic. The transcriptional activity of pGL3-6 was also normalized to that of pGL3-Basic. The activity started on construct pGL3-4, decreased on pGL3-3 and pGL3-1, and increased again on pGL3-2. Thus, the basal promoter activity was located within the -1871 to +185-bp region. The extension of the 5'-flanking sequence to -2126 bp diminished the promoter activity; however, the promoter activity increased when the 5'-flanking sequence to -1158 bp was deleted, suggesting that a negative regulatory element was located at -1871 to +185 bp and a positive regulatory element was located at -1158 to +185 bp.

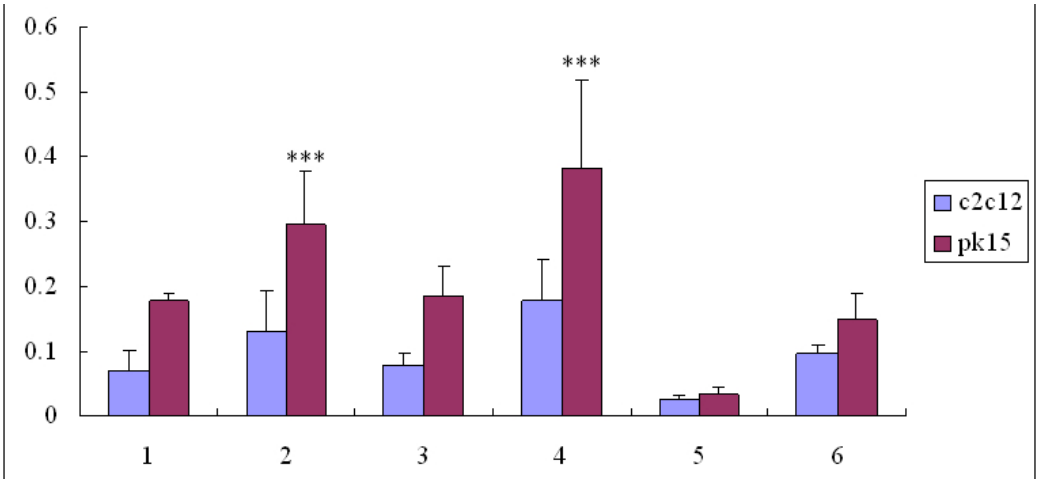

Figure 1. Transient transfection of deletion mutants of the 5'-flanking region of the porcine *PGM1* gene. The construction of the pGL reporter plasmids and transient transfection experiments are described in Methods. The luciferase activity was corrected for transfection efficiency. The results are reported as means ± SD of 3 experiments performed in duplicate. Significant levels were analyzed by the *t*-test. ***P < 0.01.

Expression profile analysis

To examine the expression of the *PGM1* gene in pigs, RNA from different tissues

(heart, liver, spleen, lung, kidney, stomach, fat, masseter muscle, small intestine, testicle, hippocampi, and pallium) was analyzed by real-time RT-PCR. The expression level of *PGM1* was normalized to that of β-actin. As expected, porcine *PGM1* was expressed in the skeletal muscle at the highest level. The second highest expression was in the liver. The gene was expressed weakly in all other tissues (Figure 2). This initial result, therefore, suggests that the *PGM1* gene is relevant in skeletal muscle.

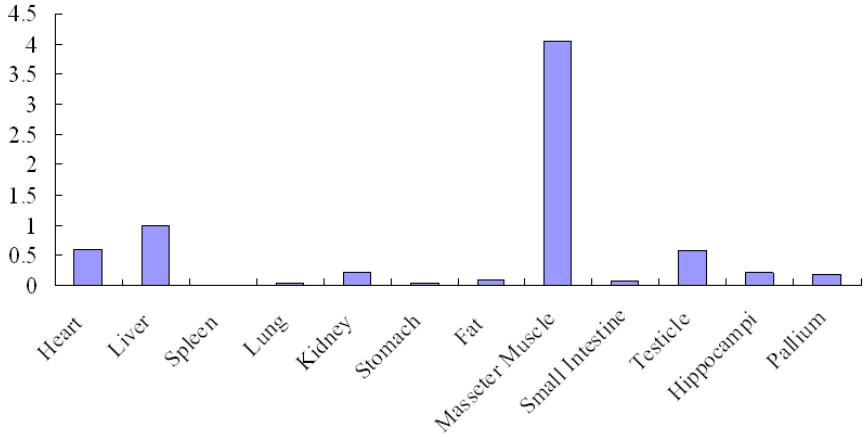

Figure 2. mRNA expression of porcine *PGM1* by real-time polymerase chain reaction. β-actin was amplified in parallel as an internal control.

PGM1 expression levels were then detected at 9 different developmental stages in Large White and Meishan pigs using real-time RT-PCR. The expression patterns of *PGM1* were different between the 2 breeds.

These analyses also indicated that the *PGM1* pattern of expression changes during skeletal muscle development. The expression of *PGM1* was high 35 and 180 days after birth (Figure 3). The Student *t*-test did not indicate a significant difference.

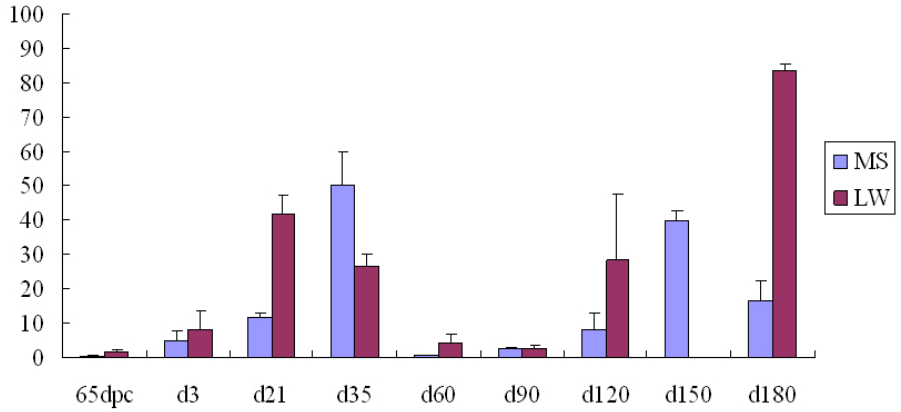

Figure 3. Expression of porcine *PGM1* in different developmental stages of longissimus dorsi was detected by real-time PCR. dpc, days post-conception; d, days after birth; MS, Meishan; LW, Large White.

PGM1 may, therefore, play an important role in the formation of muscle fibers. *PGM1* had high expression 35 and 180 days after birth (Figure 3). Surprisingly, there was almost no expression in Large White pigs 150 days after birth, but the expression level later was very significant.

Our results show that *PGM1* expression followed the same trends in Meishan and Large White pigs. Except longissimus dorsi, we also performed real-time RT-PCR analysis of samples from three 4-month-old Large White pigs to detect the expression pattern of *PGM1* in muscles containing different fibers.

As shown in Figure 3, the longissimus dorsi and biceps femoris muscles displayed a greater abundance of *PGM1* than the others; this result further implies that *PGM1* plays a critical role in skeletal muscle. There was a significant difference among the tissue types by the Student *t*-test analysis. Soleus and masseter muscles are composed mostly of type I, slow-twitch oxidative fibers, while longissimus dorsi and biceps femoris muscles contain predominantly type IIb, fast-twitch glycolytic fibers (Wang et al., 2010). *PGM1*, therefore, had high expression in type II muscle fibers.

Previous studies revealed that muscle mass is largely determined by the number of fibers, but the number of muscle fibers does not change after birth (Rowe and Goldspink, 1969; Brown, 1987). Additionally, the increase in skeletal muscle mass was mainly due to muscle fiber hypertrophy during postnatal growth, and increases in muscle mass solely through muscle fiber hypertrophy influence meat quality (Wu et al., 2011b).

In this study, *PGM1* muscle fiber expression was found to be significantly increased 35 days after birth. In the examination of 7 different muscle tissues, the longissimus dorsi and the biceps femoris had the highest *PGM1* expression levels (Figure 4), but expression in other types of muscles was not weak. Therefore, we concluded that *PGM1* plays an important role in developmental processes of muscle fibers.

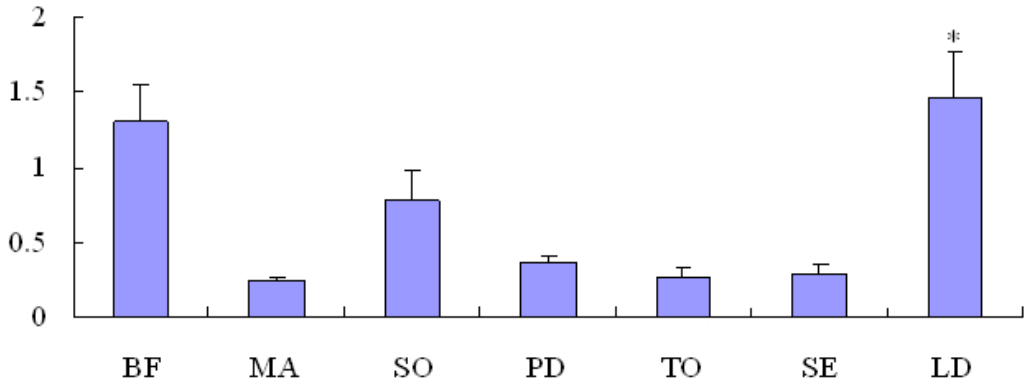

Figure 4. *PGM1* expression in 7 muscles containing different muscle fiber types in Large White pigs by real-time PCR. The expression level was normalized to that of β-actin. Results were averaged from 3 independent replicates. Error bars represent SD (N = 3). BF, biceps femoris; MA, masseter; SO, soleus; PD, Philippians dorsi; TO, toe with muscle; SE, semitendinosus; and LD, longissimus dorsi. Significant levels were analyzed by the *t*-test. *P < 0.05.

Single nucleotide polymorphism (SNP) analysis

We analyzed the genotypes and allelic frequency of the synonymous C462T mutation using PCR-restriction fragment length polymorphism, and the frequency of the C allele was

dominant in Chinese indigenous breeds.

In Table 2, the allele frequency of the *PGM1* C462T SNP was obtained by analyzing 216 pigs of different breeds. The T allele was predominant in the Large White and Landrace pigs, while the C allele was represented in Chinese indigenous pigs.

The allelic frequencies in the Chinese indigenous breeds showed a high frequency of the C allele. Table 3 reports that the C462T polymorphism did not deviate from Hardy-Weinberg equilibrium in Meishan pigs, but it did in Large White and Landrace pigs. Some important production traits were analyzed to determine their association with *PGM1* C462T. *PGM1* C462T seemed to be associated with lean meat (Table 4).

To assess the association between *PGM1* polymorphisms and economic traits in pigs, association analysis was performed using a resource population of approximately 260 F_2 individuals. Association analysis revealed that *PGM1* polymorphisms have significant associations with several meat quality traits ($P < 0.05$).

Table 2. Single nucleotide polymorphism C462T of *PGM1* among 7 pig breeds.

Breed	No. of pigs	Genotype
Large White	47	T
Landrace	29	T
Meishan	61	C
Erhualian	24	C
Qingping	28	C
Tongcheng	8	C
Jianli	19	C

Table 3. Frequency of genotypes and alleles of *PGM1*-*Taq*I restriction fragment length polymorphism.

Breed	No. of pigs	Genotype frequency and counts (observed/expected value)			Allelic frequency		χ^2 (HWE) $\chi^2_{0.05(2)} = 5.99$ $\chi^2_{0.05(1)} = 3.84$
		TT	TC	CC	T(p)	C(q)	
Meishan	61	0.02/1	0.03/2	0.9/58	0.033	0.967	14.135
Yorkshire	47	0.87/41	0.13/6	0/0	0.936	0.064	0.218
							0.003 (χ_c^2)
Landrace	29	0.72/21	0.28/8	0/0	0.862	0.138	0.743
							0.069 (χ_c^2)

$\chi^2_{0.01(2)} = 9.21$, $\chi^2_{0.01(1)} = 6.63$, $\chi^2_{0.05(2)} = 5.99$, $\chi^2_{0.05(1)} = 3.84$, $P_{TT(E)} = p^2$, $P_{TC(E)} = 2pq$, $P_{CC(E)} = q^2$.

Table 4. Statistical analysis of *Taq*I RFLP genotypes and economic traits.

Traits	*PGM1*-*Taq*I RFLP genotype (mean ± SE)			Genetic effects (mean ± SE)	
	CC	TC	TT	Additive	Dominance
Lean meat	0.540 ± 0.005[a]	0.544 ± 0.003[a]	0.556 ± 0.005[b]	0.011 ± 0.004*	0.000 ± 0.003
pH(LD)	6.387 ± 0.163	6.378 ± 0.008	6.363 ± 0.015		
pH(BF)	6.420 ± 0.014	6.424 ± 0.006	6.412 ± 0.013		
pH(SO)	6.428 ± 0.011	6.425 ± 0.005	6.420 ± 0.011		

All data in the table are least square means ± standard error. *Indicate significant differences. pH(LD), pH(longissimus dorsi); pH(BF), pH(biceps femoris); pH(SO), pH(soleus).

CONCLUSIONS

In summary, we isolated and characterized the porcine *PGM1* gene. Data presented

in our study provided an expression pattern and structural basis for future studies on *PGM1* function in porcine skeletal muscle. We have obtained evidence for a role of the *PGM1* by analyzing its expression pattern during porcine skeletal muscle formation. The expression differences in pig breeds and in different muscles indicated a possible relationship between expression and porcine meat quality and production.

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