



## Expression patterns and promoter activity analysis of *UGP2* in pigs

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**ABSTRACT.** The function of the *UDP-glucose pyrophosphorylase 2* gene (*UGP2*) in pig is not clear. In the present study, we used RNA isolated from Large White pigs and Chinese indigenous MeiShan pigs to examine the temporal coordination of changes in gene expression within muscle tissues. We cloned both the complete genomic DNA sequence and 2077-bp 5'-flanking sequence of porcine *UGP2*, to determine the genomic sequence. Real-time RT-PCR revealed that *UGP2* was highly expressed in liver and skeletal muscle of MeiShan pigs. Among different types of muscle fibers, the *UGP2* had the highest expression in both soleus muscle and longissimus dorsi in Large White pigs. In the progression of muscle fibers at different growth stages, *UGP2* plays a role in the early days after birth in Large White pigs, while in MeiShan pigs it is important later. Furthermore, the 5'-flanking sequence we cloned exhibited the promoter activity of *UGP2*, and the sequence 588 bp upstream from the transcriptional site had the greatest activity.

**Key words:** *UGP2*; Pig; Promoter activity; Expression patterns

## INTRODUCTION

Chinese indigenous pig breeds, such as MeiShan and TongCheng, often have valuable traits, including disease resistance, high fertility, good maternal qualities, and adaptability to harsh conditions, while western commercial pig breeds, such as Large White and Landrace, possess the good growth and high lean rates (Wang et al., 2012). An improved growth rate and lean meat percentage have been recognized for many years as important to strive for when trying to increase the proficiency and productivity of pig breeding (Wu et al., 2011). Although, phenotypic variances are mainly determined by the genetic differences, our understanding of the molecular events controlling muscle synthesis in pigs was still rudimentary. Evaluation of the genetic or expression differences between Chinese indigenous pig breeds and western commercial pig breeds that are responsible for these phenotypic variances was, therefore, necessary.

The *UDP-glucose pyrophosphorylase* gene (*UGP2*) was isolated by fluorescence *in situ* hybridization, and assigned to porcine chromosome 3q21-q22 following analysis of somatic cell and radiation hybrid panels (Looft et al., 2000). Some recent research has described the effects of *UGP2* on the reduced frequency of milk within the bovine mammary gland (Littlejohn et al., 2010; Mohammad et al., 2012).

Our present study used the mRNA differential display technique (Liang and Pardee, 1992) to analyze porcine *UGP2* and identify differentially expressed genes of muscle tissues between Chinese indigenous MeiShan and Large White pigs.

## MATERIAL AND METHODS

### Animals

All pigs were fed at the Jingpin Pig Station of the Huazhong Agriculture University (Wuhan, China) and all animal procedures were performed according to protocols approved by the Hubei Province, P.R. China Biological Studies Animal Care and Use Committee.

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

For expression analysis of different muscle types, fast twitch and oxidative masseter and soleus were collected from three 120-day-old Large White pigs. Adult heart, liver, spleen, lung, kidney, stomach, muscle, fat, ovary, uterus, and brain samples, and embryonic heart, liver, spleen, lung, kidney, stomach, muscle, and brain samples were also collected, from a female, 150-day-old indigenous MeiShan pig and a MeiShan pig embryo, respectively, for spatial expression analysis.

### Bioinformatic analysis

The open reading frames (ORF) for translated peptides were predicted using NCBI's online ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and DNASTAR. ClustalW (<http://www.ebi.ac.uk/clustalw/>) was used for multiple sequence alignment and shading was done using BOXSHADE 3.21 (<http://www.ch.embnet.org>). Furthermore, the ProScan software (version 1.7) was used to predict putative promoter and transcription factor binding sites (<http://www-bimas.cit.nih.gov/molbio/proscan/>).

## RNA extraction and cDNA preparation

Total RNA were isolated from frozen pig tissues (heart, liver, spleen, lung, kidney, stomach, fat, masseter muscle, small intestine, testicle, hippocampi, and pallium) using Trizol reagent (Invitrogen, USA). Each sample was treated with RNase-free DNase (Promega, USA) to remove contaminating genomic DNA. The integrity of RNA samples was analyzed by ethidium bromide staining of ribosomal RNAs on 1% formaldehyde denaturing agarose gels, and the quality was assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), with an optimal 260/280 ratio being between 1.8 and 2.1. cDNA was synthesized from 1 µg total RNA in 20 µL reactions using a RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, Canada). The result cDNA was then diluted 5 to 10 times with double-distilled water and stored at -20°C.

## Plasmid construction

Primers were designed according to the strategy of a 5'-end deleted mutation of the promoter region, with marked *Kpn* and *Xho* restriction sites at the 5'-ends of forward and reverse primers, respectively (Table 1). PCR-amplified products were first double-digested and then subcloned into the pGL3-Basic plasmid (Promega), and subsequently sequence.

**Table 1.** Primers used in the present study.

Primer name	Primer sequence (5'-3')	Size (bp)	Tm (°C)
<i>UGP2</i> quantitative real-time PCR			
PM1a-F	CATCACATGAGTTTGAGCACACTAA	145	59
PM1a-R	GATTTTTCCCCAGTCCACAGAA		
<i>UGP2</i> sequence amplification			
U-1F	CGGGGTACCAAGCACAAAATAAGACTG	2077	60
U-2F	CGGGGTACCGAACTCATAAGTAGGCAG	1759	60
U-3F	CGGGGTACCATTCTGGAAGGCTTTACG	1518	60
U-4F	CGGGGTACCATCCAAGCAATCAAAGA	1403	60
U-5F	CGGGGTACCCAGGTTTCAGTCTTGCGT	961	60
U-6F	CGGGGTACCTTCCAGTATGTCATCACC	884	60
U-7F	CGGGGTACCGTGCTCTCTTCAACAAC	588	60
U-R	CCGCTCGAGGTCCAACAATCCACAAC		60
β-actin			
β-actin-F	CCAGGTCATCACCATCCGG	158	58
β-actin-R	CCGTGTTGGCGTAGAGGT		

## Cell culture, transient transfection, and luciferase assays

Pig kidney cells (PK-15) and C2C12 myoblast cells purchased from CCTCC (China Center for Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Gibco, USA), supplemented with 10% (v/v) bovine calf serum (Gibco), on coverslips of 24-cell plates at 37°C with humidified air containing 5% CO<sub>2</sub>. When cells reached 60-70% confluence, transient transfections were carried out using Lipofectamine™ 2000 (Invitrogen), according to manufacturer instructions. The medium was changed when 360 min after transfection and then cells were incubated for another 1440 min. The pGL3-Control vector was used as a positive control, the phRL-TK plasmid was co-transfected into PK-15 and C2C12

for recombinant construction, pGL3-Basic and pGL3-Control plasmids were co-transfected into PK-15 for recombinant construction, pGL3-Basic and pGL3-Control plasmids were used as internal controls.

Extracts from transfected cells were collected following rocking for 20 min with 1X passive lysis buffer (Promega). The relative activity of the 7 fragments of *UGP2* was measured with a TD-20/20 luminometer using the Dual-Luciferase (firefly and *Renilla* luciferase) Reporter Assay System (Promega). Analysis of each fragment was repeated three times. The Student *t*-test was performed to compare the transcriptional activity among these recombinants, and  $P < 0.05$  was considered to be significant.

### Real-time reverse transcription (RT-PCR) analysis

Real-time RT-PCR was performed with a Lightcycler 480 (Roche, France). All reactions were done in triplicate in a total volume of 20  $\mu$ L each. Each 20- $\mu$ L real-time RT-PCR included 10  $\mu$ L Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR SuperMix-UDG (Invitrogen), 1  $\mu$ L cDNA, and 0.5  $\mu$ L primers. Initial denaturation was performed at 95°C for 2 min, followed by 45 cycles of 95°C for 20 s, 59°C for 20 s, and 72°C 20 s, with fluorescence recording at 72°C. The Gene Expression Macro software (ABI, USA) was used for an optimized comparative Ct ( $2^{-\Delta\Delta Ct}$ ) value method (Livak and Schmittgen, 2001). The  $2^{-\Delta\Delta Ct}$  equation is valid only if the amplification efficiencies of control and target genes are approximately equal. Table 1 lists the primers used in this study. The Student *t*-test was conducted to identify genes differing in expression,  $P < 0.05$  was considered to be significant.

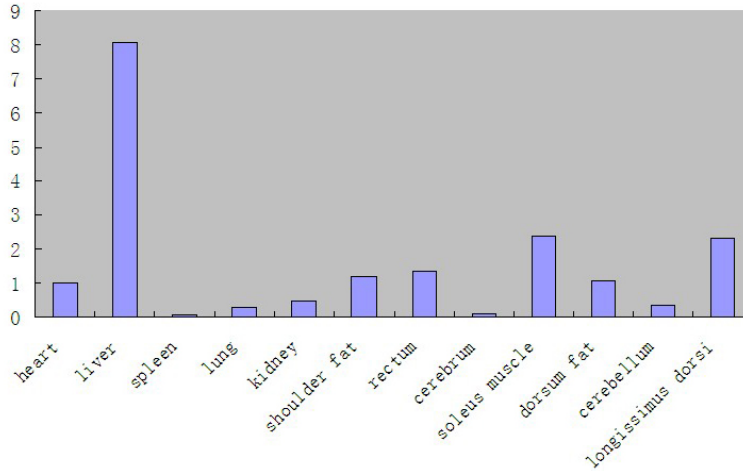
## RESULTS AND DISCUSSION

### Expression profile analysis

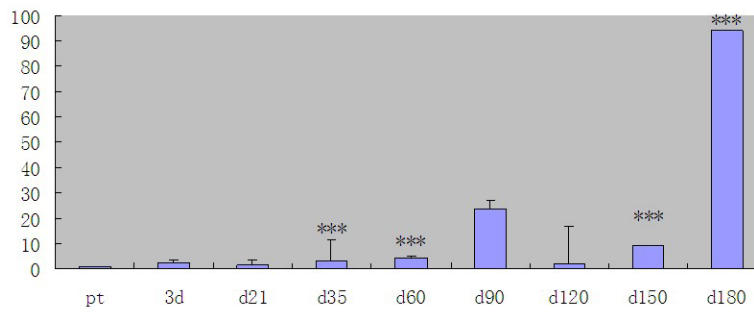
In this study, the complete genomic DNA sequence and fundamental structure of the porcine *UGP2* gene was determined. Using bioinformatic methods, porcine *UGP2* was compared with the gene sequence of human, mouse, and rat *UGP2*, which consisted of 10 exons and 9 introns. Furthermore, exon-intron junctions followed the GT-AG rule for splice-donor and splice-acceptor sites. The nucleotide sequence of porcine *UGP2* cDNA shared 99, 89, 99, and 73% identity with *UGP2* of human, mouse, cattle, and chicken origin, respectively.

To examine the expression of the *UGP2* gene in pigs, RNA from various 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 *UGP2* was normalized to  $\beta$ -actin expression. As expected, porcine *UGP2* was expressed at the highest level in liver tissues, and second highest in skeletal muscle. The gene was expressed weakly in all other tissues (Figure 1). The initial result, therefore, suggests that the *UGP2* gene is relevant with the skeletal muscle.

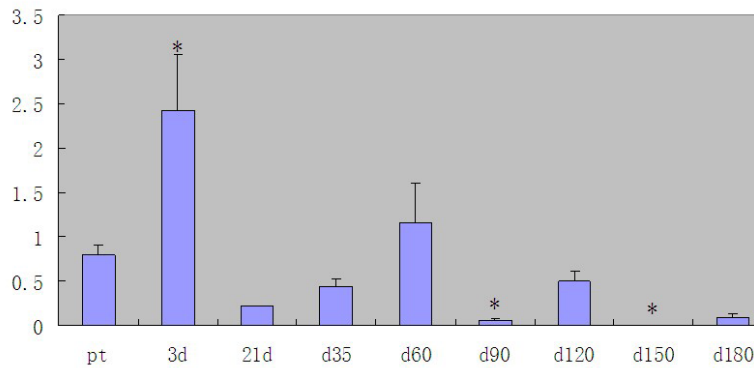
*UGP2* expression levels were then detected at 9 different developmental stages in Large White and MeiShan pigs using real-time RT-PCR. The expression patterns of *UGP2* were different between the two breeds (Figures 2 and 3).



**Figure 1.** mRNA expression of porcine *UGP2* by real-time PCR.  $\beta$ -actin was amplified in parallel as an internal control.



**Figure 2.** Expression of porcine *UGP2* in different developmental stages of longissimus dorsi was detected by real-time PCR in Chinese indigenous Meishan pig. pt = 65 days post-conception; d3, d21, d35, d60, d90, d120, d150, d180 = days after birth. Significant levels were analyzed by the *t*-test. \*\*\* $P < 0.01$ .



**Figure 3.** Expression of porcine *UGP2* in different developmental stages of longissimus dorsi was detected by real-time PCR in Large White pig. pt = 65 day post-conception; d3, d21, d35, d60, d90, d120, d150, d180 = days after birth. Significant levels were analyzed by the *t*-test. \* $P < 0.05$ .

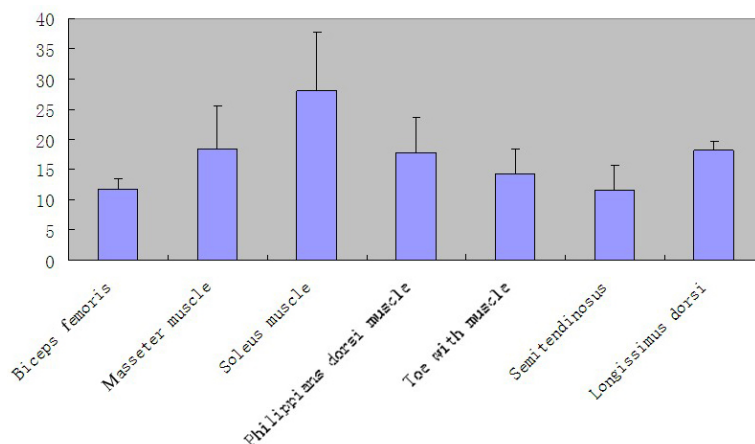
These analyses also concluded that the *UGP2* pattern of expression changes during skeletal muscle development. The expression of *UGP2* was the highest at 180 days after birth in MeiShan pigs (Figure 2) and Student *t*-tests revealed significant differences in *UGP2* expression between the ages of 35, 60, 150, and 180 days. *UGP2* may, therefore, play an important role after birth in Chinese indigenous pigs. Conversely, *UGP2* expression was higher in younger Large White pigs (Figure 3), highest in pigs aged 3 days and then less with increasing age, with significant differences among pigs aged 3, 90, and 150 days identified with Student *t*-tests. Taken together, these findings suggest that *UGP2* plays a role in the early days after birth in western commercial pigs, while in Chinese indigenous pigs it is important later.

In pigs, primary myofibers are formed after 35 to 55 days of gestation, and secondary fibers are formed between 55 and 90 to 95 days of gestation, whereas the total number of muscle fibers is generally considered to be established definitively by 90 to 95 days of gestation (Ashmore et al., 1973; Wigmore and Stickland, 1983).

Our results show that *UGP2* expression is different between MeiShan and Large White pigs. *UGP2* may be more important in the formation of muscle fibers in MeiShan pigs, while it may be involved in primary fiber formation in Large White pigs, as it gradually increased from day 21 to day 60 and then decreased in the latter.

Except for longissimus dorsi, we also performed real-time RT-PCR analysis of samples from three individual 4-month-old Large White pigs to detect the expression pattern of *UGP2* in muscles containing different fibers.

As shown in Figure 4, the soleus and longissimus dorsi muscles displayed a greater abundance of *UGP2* than the others, a result that further implies that *UGP2* plays a critical role in skeletal muscle. There was not 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). *UGP2*, therefore, had a high expression in type I and II muscle fibers.



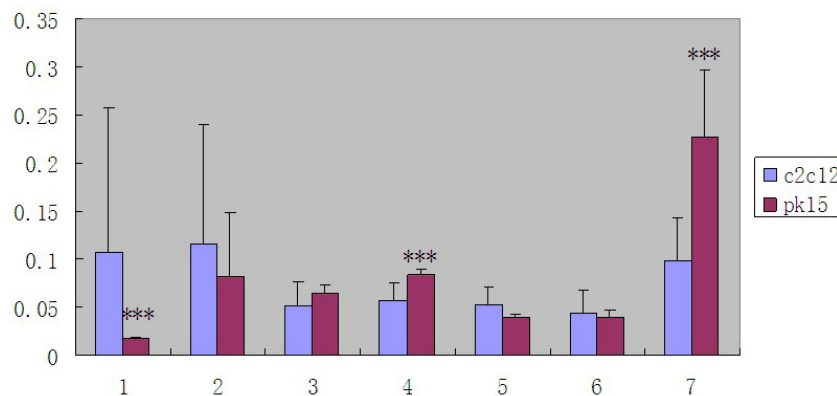
**Figure 4.** *UGP2* expression in seven different muscles that contained different muscle fiber types in Yorkshire pigs by real-time PCR. The expression level was normalized to  $\beta$ -actin. Results were from three independent replicates. Error bars represent SD (N = 3). Seven different muscles: biceps femoris (1), masseter muscle (2), soleus (3), Philippians dorsi muscle (4), toe with muscle (5), semitendinosus (6), longissimus dorsi (7) were analyzed.

Previous studies revealed that muscle mass is largely determined by the number of fibers, whereas the number of muscle fiber number does not change after birth (Rowe and Goldspink, 1969; Brown, 1987).

In the present study, *UGP2* muscle fiber expression was different between MeiShan and Large White pigs. In the examination of seven different muscle tissues, the soleus and longissimus muscles had the highest *UGP2* expression levels, but expression in other types of muscles was also strong (Figure 4). We, therefore, conclude that *UGP2* plays an important role in developmental processes of muscle fibers.

### Promoter activity analysis

The promoter of porcine *UGP2* (NCBI Reference Sequence: NM\_213980.1) was predicted, which was 2077-bp 5'-flanking region before the transcription site. To determine the location of *UGP2* promoter activity, we studied the transcriptional activity of recombinants containing progressive 5'-deletion DNA fragments (2778/701) linked to a pGL3 reporter. There were 7 fragments in total: 1) from 2778 to 701 bp, 2) from 2778 to 1019 bp, 3) from 2778 to 1260 bp, 4) from 2778 to 1375 bp, 5) from 2778 to 1817 bp, 6) from 2778 to 1894 bp, and 7) from 2778 to 2190 bp. The recombinants were transiently transfected into PK-15 and C2C12 cells. The activity started on construct 701, was the strongest on (7) 2190 and the lowest on (1) 2778 (Figure 5). The basal promoter activity, therefore, was located within 2778 to 2190 bp, whereas the upstream 588 bp conferred maximal transcriptional activity. The extension of the 5'-flanking region up to 2778 diminished promoter activity. The results revealed that a 588-bp fragment located before the start site ATG had the highest activity in C2C12 and PK-15 cells. With *t*-tests, significant differences among the first fragment, the fourth fragment, and the last fragment were identified in PK-15 cell. The promoter activity of *UGP2* was more clearly seen in PK-15 cells likely because they are pig kidney cells of pig.



**Figure 5.** Transient transfection of deletion mutants of the 5'-flanking region of the porcine *UGP2* gene. Description of the construction of the pGL-Ibs reporter plasmids and transient transfection experiments were described in Methods. Luciferase activity was corrected for transfection efficiency with the values obtained with *Renilla*. Results are mean SD of three experiments performed in duplicates. Significant levels were analyzed by the *t*-test. \*\*\**P* < 0.01. For numbers 1-7, see legend to Figure 4.

In summary, our study provided the first insight into the molecular characteristics and promoter activity of *UGP2* in pigs. Our findings should be helpful in the investigation of *UGP2* functions in porcine muscles, which may ultimately contribute to the elucidation molecular mechanisms behind skeletal muscle formation.

## ACKNOWLEDGMENTS

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