



Molecular Cloning and mRNA Expression of the Porcine Insulin-responsive Glucose Transporter (GLUT4)*

Jianjun Zuo^a, Fawen Dai^a, Dingyuan Feng^{**}, Qingyun Cao, Hui Ye, Zemin Dong and Weiguang Xia

College of Animal Science, South China Agricultural University, Guangzhou, Guangdong Province, 510642, China

ABSTRACT : Insulin-responsive glucose transporter 4 (GLUT4) is a member of the glucose transporter family and mainly presents in skeletal muscle and adipose tissue. To clarify the molecular structure of porcine GLUT4, RACE was used to clone its cDNA. Several cDNA clones corresponding to different regions of GLUT4 were obtained by amplifying reverse-transcriptase products of total RNA extracted from Landrace porcine skeletal muscles. Nucleotide sequence analysis of the cDNA clones revealed that porcine GLUT4 cDNA was composed of 2,491 base pairs with a coding region of 509 amino acids. The deduced amino acid sequence was over 90% identical to human, rabbit and cattle GLUT4. The tissue distribution of GLUT4 was also examined by Real-time RT-PCR. The mRNA expression abundance of GLUT4 was heart>liver, skeletal muscle and brain>lung, kidney and intestine. The developmental expression of GLUT4 and insulin receptor (IR) was also examined by Real-time RT-PCR using total RNA extracted from *longissimus dorsi* (LM), *semimembranosus* (SM), and *semitendinosus* (SD) muscle of Landrace at the age of 1, 7, 30, 60 and 90 d. It was shown that there was significant difference in the mRNA expression level of GLUT4 in skeletal muscles of Landrace at different ages ($p < 0.05$). The mRNA expression level of IR also showed significant difference at different ages ($p < 0.05$). The developmental change in the mRNA expression abundance of GLUT4 was similar to that in IR, and both showed a higher level at birth and 30 d than at other ages. However, there was no significant tissue difference in the mRNA expression of GLUT4 or IR ($p > 0.05$). These results showed that the nucleotide sequence of the cDNA clones was highly identical with human, rabbit and cattle GLUT4 and the developmental change of GLUT4 mRNA in skeletal muscles was similar to that of IR, suggesting that porcine GLUT4 might be an insulin-responsive glucose transporter. Moreover, the tissue distribution of GLUT4 mRNA showed that GLUT4 might be an important nutritional transporter in porcine skeletal muscles. (**Key Words :** GLUT4, IR, Skeletal muscle, Porcine)

INTRODUCTION

In mono-gastric animals such as pigs, dietary carbohydrate is converted to single glucose and absorbed directly in intestines. Most of glucose is brought to skeletal muscles with constant flow of blood and preserved as glycogen to provide energy for activity and metabolism of muscle. Glucose is the main energy source in the porcine skeletal muscle tissue. Thus, it is very important for the absorption, transport and metabolism process of glucose. A recent research further confirmed that there is a positive

linear association between muscle glycogen concentration at a slaughter and the rate of pH decline (Ferguson et al., 2008). Since the rate and extent of pH decline during the conversion of muscle to meat will significantly impact meat quality attributes (Scheffler and Gerrard, 2007), glucose metabolism in skeletal muscle is very important for meat quality in pigs.

Facilitated diffusion of glucose across the plasma membrane is mediated by glucose transporter protein. To date, at least thirteen isoforms of GLUT have been studied (Joost et al., 2002; Zhao and Keation, 2007). GLUT4 (SLC2A4, facilitated glucose transporter, member 4) is the mainly glucose transporter expressed in skeletal muscle. GLUT4 plays a key role in cellular glucose uptake stimulated by insulin in these cells, and thereby is called insulin-responsive GLUT. In myocytes, GLUT4 is associated with membrane structure and recycled between the plasma membrane and the intracellular tubulovesicular pool. The understanding of signaling pathways utilized by

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** Corresponding Author: Dingyuan Feng. Tel: +86-20-852 86090, Fax: +86-20-85286090, E-mail: fengdy@scau.edu.cn

^a These two authors contribute equally to this study.

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insulin has been extensively reviewed (He et al., 2007; Watson and Pessin, 2007). To regulate glucose uptake, the insulin receptor (IR) proteins undergo tyrosine phosphorylation in response to insulin stimulation and are known to play key roles in insulin-stimulated glucose uptake in muscle (Saltiel and Pessin, 2002). It has been shown that IR was expressed in hamster preimplantation embryos from the morula stage onwards while GLUT4 was not detected at mRNA level (Tonack et al., 2009). The development evaluation of GLUT4 and IR allows assessment of which mechanisms (i.e. insulin sensitivity or GLUT4 content) are defective in skeletal muscle tissue from pigs.

Although the structures of GLUT genes in humans and rats have been widely investigated and well established, little information is available for pigs, except for the partial cDNA sequence of porcine GLUT4. In the present study, porcine GLUT4 cDNA was cloned by RACE method, and the tissue distribution and developmental expression pattern of GLUT4 mRNA in Landrace were conducted by Real-time RT-PCR analysis.

MATERIALS AND METHODS

Animal and sample collection

A total of 25 purebred Landrace gilts were divided into five groups at the ages of 1, 7, 30, 60 and 90 d, respectively. The piglets were weaned at the age of 28 d. All procedures were approved by the Animal Care Committee at the South China Agricultural University. The animals used in this experiment were cared for in accordance with the guidelines established by University Council of Animal Care. Skeletal muscle tissue samples (*longissimus dorsi* muscle (LM),

semimembranosus (SM), and *semitendinosus* (SD) muscle)) were collected from a total of 25 pigs at different ages: suckling (1, 7 d) and post-weaning (30, 60, 90 d), and 5 pigs were sacrificed at each day. Moreover, heart, liver, skeletal muscle, brain, lung, kidney and intestine (duodenum and jejunum) tissue samples were gained from pigs at the age of 7 d.

cDNA cloning of porcine GLUT4 by RACE

3'/5' RACE primers were designed as Table 1, based on the partial cDNA sequence of porcine GLUT4 (Genebank NO. AF184171).

For 3' RACE, cDNA was synthesized from total RNA isolated from porcine skeletal muscle, with using 3'RACE CDS primer and MMLV reverse transcriptase, according to the manufacturer's instructions of RACE cDNA Amplification kit (Clontech, USA). First PCR was carried out with the cDNA, GSP2A/B primer and UPM primer. Second PCR was carried out with the products of the first PCR, NGSP2A/B primer and NUP primer. For 5'RACE, cDNA was also synthesized from total RNA isolated from porcine skeletal muscle, with using 5'RACE CDS primer, SMART II A primer and MMLV reverse transcriptase. First PCR was carried out with the cDNA, GSP1 primer and UPM primer. Second PCR was carried out with the first PCR products, NGSP1 primer and NUP primer. Those second PCR products were recovered and purified. Then they were inserted into pGEM-T vector (Invitrogen), transformed into DH5 α *E. coli* and sequenced.

The open reading frame (ORF) PCR was conducted to improve the reliability of sequencing results of RACE. The ORF PCR was carried out with mix cDNA of skeletal muscles. Details of primer design of ORF PCR are

Table 1. Sequences of oligonucleotide primers used for RACE and ORF PCR

Type	Sequences
3'RACE CDS primer	5'-AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ N ₁ N-3' (N = A, C, G, or T; N-1 = A, G, or C)
5'RACE CDS primer	5'-(T) ₂₅ N ₁ N-3'(N = A, C, G, or T; N-1 = A, G, or C)
SMART II A	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
Universal primer (UPM)	Long: 5'-CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT-3' Short: 5'-CTAATACGACTCACTATAGGGC-3'
NUP primer	5'-AAGCAGTGGTATCAACGCAGAGT-3'
GSP1 primer	5'-GAGGGTGGTGAGGGTGCCTGGTG-3'
NGSP1 primer	5'-CGGAGAAGACGGCGAGGACAA-3'
GSP2A primer	5'-CCTACGAGATGCTCATTCTTGGACGG-3'
NGSP2A primer	5'-GTGCCTTGGGGACGCTCAACC-3'
GSP2B primer	5'-CCTGATGACTGTGGCTCTGCTTCTGC-3'
NGSP2B primer	5'-GCCCCATCCCCTGGTTCATC-3'
ORF sense primer	5'-ATGCCGTCGGGCTTCCAA-3'
ORF antisense primer	5'-GCCCCCTCAGTCATTCTCATCG-3'

presented in Table 1. These PCR products were electrophoresed on a 1% agarose gel, recovered and purified. Then they were inserted into pGEM-T vector (Invitrogen), transformed into DH5 α *E. coli* and sequenced.

DNA sequence analysis

The analysis of cDNA sequence was conducted with the computer programs of DNASTar and the National Center for Biotechnology Information (NCBI) BLAST site. The multiple sequence alignment was performed with Vector NTI Suit 5.5. The transmembrane domain was predicted according to the deduced amino acids of GLUT4 using the program on the HMMTOP version 2.0 (<http://www.enzim.hu/hmmtop/>).

Real-time RT-PCR analysis

Total RNA was isolated from 100 mg of tissue samples using TRIZOL reagent (Invitrogen) and purified with DNase I (Invitrogen) according to the manufacturer's instructions. The RNA quality was checked using 1.0% agarose gel electrophoresis and stained with 0.5 μ g/ml ethidium bromide. The RNA had an OD260nm: OD280nm ratio between 1.8 and 2.0. Synthesis of the first strand of cDNA was performed with oligo(dt)20 and Superscript II reverse transcriptase (Invitrogen).

Real-time PCR was performed using one-step SYBR Green PCR Mix (Takara, Dalian, China), containing MgCl₂, dNTP, and Hotstar Taq polymerase. Primers were designed specifically for GLUT4 and IR by using Primers 5.0 software. Amplification and melting curve analysis was performed by ABI 7500 (Applied BioSystems). Melting curve analysis was conducted to confirm the specificity of each product and the size of the product were verified on ethidium bromide-stained 1.0% agarose gels in Tris acetate-EDTA buffer. The relative mRNA expression was calculated by $2^{-\Delta\Delta C_t}$ ($\Delta C_t = C_t$ of the target gene - C_t of the housekeeping gene) (Livak and Schmittgen, 2001), and β -actin was taken as internal reference gene in this study. Real-time PCR efficiency was acquired by the amplification of serial dilution of plasmids contained target fragment according to the equation $10^{(-1/\text{slope})}$ and kept consistent between target genes and β -actin. Negative controls were performed in which water was substituted for cDNA. Details of primer design and runs of real time RT-PCR are presented in Table 2.

Statistical analysis

All the results were processed with SAS V8 (The SAS Institute, Cary, NC). The influence of ages (1, 7, 30, 60 and 90 d) and skeletal muscle tissues (LM, SM and SD) were analyzed as 5 \times 3 factor design. Statistical analysis of tissue distribution of GLUT4 mRNA was performed with a one-way ANOVA, followed by LSD test. In all cases, $p < 0.05$ was considered to be statistically significant different. The results were presented as means \pm SEM.

RESULTS

Cloning of porcine GLUT4

The porcine GLUT4 cDNA was amplified by RACE and sequenced for the complete nucleotide and deduced amino acid sequences. As shown in Figure 1, porcine GLUT4 cDNA consisted of 2,491 bp of nucleotide with 34 bp of 5' flanking un-translated sequence, followed by 1,530 bp of open reading frame coding 509 amino acids, and 927 bp of 3' flanking sequence. We further checked our RACE results by the amplification of open reading frame (ORF). As shown in Figure 2, the expected bands were gained by ORF PCR, and the sequencing results of ORF were also same as these of RACE, which improved the reliability of amplified sequence of porcine GLUT4 with RACE method.

Sequence analysis of porcine GLUT4

The deduced amino acid sequence of porcine GLUT4 was compared and aligned with that of other three mammalian species: human (Genebank No. M20747), rabbit (Genebank No. NM001089313), and cattle (Genebank No. D63150). As shown in Table 3, these four mammalian animals have the same deduced amino acid number, 509 amino acids. Moreover, the deduced amino acid sequence was 94.5%, 95.1% and 93.1% identical to human, rabbit and cattle GLUT4, respectively.

In this study, we compared the deduced transmembrane domains of porcine GLUT4 with other species such as human, rabbit and cattle, and the results were shown in Figure 3. Interestingly, porcine GLUT4 had same orientation in the plasma membrane as that of other species. All of them hold 12 transmembrane domains. In addition, porcine GLUT4 is highly homologous with that of other species.

Table 2. Details of primer design and runs of GLUT4, IR and β -actin

Gene	Primer sequence	Product (bp)	Anneal temperature
GLUT4	S: 5'-CTTCCTGCTTGGCATCATCT-3'	146	58°C
	As: 5'-TGAGGAACCGTCCAAGAATG-3'		
IR	S: 5'-GGAAACGCCAGGGACAT-3'	235	58°C
	As: 5'-GGGAACGCAGGTAAGTCTT-3'		
β -actin	S: 5'-CATCACCATCGGCAACG-3'	152	58°C
	AS: 5'-CCGTGTTGGCGTAGAGGT-3'		

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1  GGTGGGCAACAGCCAGCTTCTGCTAAGCAACATGCGGCTGGGCTTCCACAGATCCG
1      M P S C F Q Q I C
61  CTCGAAAGATGGGAGCCCTCAGCAGGAGTCACTGGGACCTTGTCTCGGCGCTCTI
10   S E D G E P P Q Q R V T C T L V L A V F
121  CTCGGCTGTGCTGGCTCCCTGAGTTTGGCTACACATTTGGCTCATCAAAGCCGCCA
30   S A V L G S L Q F G Y N I G V I N A P Q
181  GAAGGTGATTGAACAGAGCTACAATGAGAGTGGCTGGGAGGCAAGGGCCAAATGGACC
50   R V J E Q S Y R E T W L G R Q G P N C P
241  CGGCTCCATGACACAGGACCCCTGCTACDCTTGGCTCTCTGGTGGCCATCTCTCTC
70   G S I P P G T L T L T L N A L S V A I F S
301  TGTGGTGGATGTTCTCTCTCTGCTGGCTGGCTCTCTCTCTCTCTCTCTCTCTCTCT
90   V G G M F S S F L L G I V S Q W L G R K
361  GAAGGCAATGCTGTCATTAACACDCTGGCAGTGGCTGGGCTGGCTGGCTGGCTGGCT
110  R A M L F N R T L A V I A C A L M G I A
421  CAAGCGGCTGCTCTAGAGAGTGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
130  K A A A S Y E M L I L C R F L I C A Y S
481  AGGCTGGCATCTGGCTGCTGGCTGCTGGCTGCTGGCTGCTGGCTGCTGGCTGCTGGCT
150  G L A S G L V P W Y V G E I A P I H L R
541  GGTGGCTGGGAGACTCAAGGAGCTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
170  G A L G T L N Q L A I V I C I L I A Q V
601  GCTGGCTGGGAGTGTCTGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGG
190  I G L E S M L C I A I I N P L L L G I T
661  AGTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGG
210  V L P A L L Q L V L L P F C P E S P R Y
721  CCTCTATATCATCGGAGCTAGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
741  L Y I I R N L E G C P A R K S I K R L T I
781  CTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
250  W A D V S G A L A E L K E E K R Q L E M
841  TGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
270  E R P L S L L Q L L G S H T H R Q P L I
901  CATTGCATTTGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGG
310  I A I V L Q L S Q Q L S C I N A V F Y Y
961  TTCAGAGCATCTGTGAGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
330  B T S I F T L N Q L A I V I C I L I A Q V
1021  CGTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGG
350  G V V N T V F T L V S V L L V E R A G R
1081  CCGGAGCTCCATCTCTGGGCTGGGAGGAATGTGGCTGGCTGGCTGGCTGGCTGGCTGG
1141  R T L H L L G L A G M C G C A I L M T V
370  GGTCTGCTTCCGCTGGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
1201  A L L P L E R V P A N S Y V S I V A I F
390  TGGCTTCTGGCTTCTTTCAGATGGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
1261  G C T C T T C A G C A G G G C C C C C C C C C C C C C C C C C C C C C C
410  L F S Q G C P R P A A M A Y A G F S K W I
1321  GTCACACTTCATATCCGGATGAGTTCACAGTATGTTCGGATGCTATGGGGCCCTAGCTI
430  S N P J I G M S F Q Y V A D A M G P Y V
1381  CTCTCTCTCTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
450  P L L F A V L L L G F P I F T F L K V P
1441  TGAACCGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
470  E T R G R T F D Q I S A T F R R T P S L
1501  TTTAGAGCAGGAGTGAACCCAGCAGAACTCGAGTACTTAGGGCCAGATGAGAATGA
490  L E Q E V K P S T R L R Y I G P D R N D
1561  CTAAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
1621  *
1681  *
1821  TTTGCTCTGACGACTTAAKCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
1881  GCAGCCTGCGGATGGGAGCTAGAGGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
1741  CTGTGACTCTGGATATTATGTGTGTGGTGGCTGGGGGGGGGGGGGGGGGGGGGGGGGG
1801  CCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
1861  CAGCCCTGAGCCAGGGAGCGGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
1921  GGGAGCTTACGGAGCGAGCAGCAGGACTGGAGGAGAAATCCAGTTCTCTGCTCTCTGG
1981  GCACTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
2041  GGGTCTGTCTTGGCTCTCAGGGCAGAGGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
2101  GCTGAGGCTCAGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
2161  CTAGACTCAGGGCTCTGGGGCCACTCGGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
2221  CCGGGGGGGGGAGAGGGAGCAGACTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
2281  TCCCCAGGGCTTGGCCACTAAGGAAAGAGGGGATTTGAAGGCTGCTGTGAACAC
2341  GGGCTGGAGGAGCTCTGAGATAHTTTGTATATGTTGAAGGAGGGGGGGGGGGAGAA
2401  GAAACCGAAGGCTCTGCTACTAAATGATAGAGAGCTCCGTATATAAGTCACTGTA
2461  AGAGAAAAAAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
    
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Figure 1. The nucleotide sequence and deduced amino acid sequence of porcine GLUT4. * Indicates stop code.

Distribution of GLUT4 mRNA in porcine tissues

To examine the distribution of GLUT4 in different porcine tissues, we detected the mRNA expression in heart,

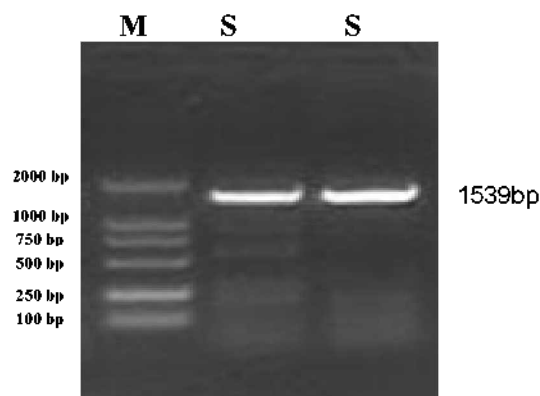


Figure 2. The amplification of open reading frame. M: marker, S: sample of skeletal muscles.

liver, skeletal muscle, brain, lung, kidney and intestine by Real-time RT-PCR. Figure 4 showed the tissue distribution pattern of porcine GLUT4. The highest mRNA expression of GLUT4 was observed in heart and skeletal muscle, which was significantly higher than that in other tissues ($p > 0.05$). The liver showed significantly higher level of GLUT4 mRNA expression than that in brain, lung, kidney and intestine ($p > 0.05$). The lowest expression of GLUT4 mRNA was detected in kidney, intestine and lung.

Development of GLUT4 and IR mRNA expression in porcine skeletal muscle

The current study was conducted to examine the development of GLUT4 mRNA in Landrace skeletal muscle at the age of 1, 7, 30, 60 and 90 d (Figure 5). It was shown that there was significant difference in the relative mRNA expression of GLUT4 in skeletal muscle from Landrace pigs at different ages ($p = 0.016$). The relative mRNA expression of GLUT4 in skeletal muscles was higher at the age of 1 and 30 d than that at the other ages. The relative expression level of GLUT4 from LM muscle at the age of 1d was significantly higher than those at the age of 7, 30, 60 and 90 d ($p < 0.05$). Both of SM and SD showed significantly higher expression of GLUT4 at the age of 30 d than those at the age of 7 d ($p < 0.05$). However, there was no significant difference in GLUT4 mRNA expression level among different skeletal muscle tissues ($p > 0.05$). It also was not detected significant interaction between age and tissue for GLUT4 mRNA expression level in this study.

Table 3. Amino acid identity between porcine GLUT4 and other mammalian GLUT4

GLUT4	Identity (%)	Predicted amino acid number
Porcine		509
Human	94.5	509
Rabbit	95.1	509
Cattle	93.1	509

Pig	MPSGFQQIGS EDGEPPQQRV	TGTLVLA VFS AVLGSLQFGY NIGV	NAPQK	50	
Human	MPSGFQQIGS EDGEPPQQRV	TGTLVLA VFS AVLGSLQFGY NIGV	NAPQK	50	
Rabbit	MPSGFQQIGS EDGEPPQQRV	TGTLVLA VFS AVLGSLQFGY NIGV	NAPQK	50	
Cattle	MPSGFQQIGS EDGEPPRQRV	TGTLVLA VFS AVLGSLQFGY NIGV	NAPQK	50	
Pig	VIEQSYNETW LGRQGPNGPG SIPPGT	LTTL WALSV AIFS V GGMFSSFLIG		100	
Human	VIEQSYNETW LGRQGPNGPG SIPPGT	LTTL WALSV AIFS V GGMFSSFLIG		100	
Rabbit	VIEQSYNETW LGRQGPNGPG SIPPGT	LTTL WALSV AIFS V GGMFSSFLIG		100	
Cattle	VIEQSYNETW LGRQGPNGPG SIPPGT	LTTL WALSV AIFS V GGMFSSFLIG		100	
Pig	IVSQWLGRKK AMLFNNTLAV LAGALMGLAK	AAASYEM LIL GRFLIGAYSG		150	
Human	IVSQWLGRKR AMLVNNVLAV LGGSLMGLAN	AAASYEM LIL GRFLIGAYSG		150	
Rabbit	IVSQWLGRKR AMLANNVLAV LGGTLMGLAN	AAASYEM LIL GRFLIGAYSG		150	
Cattle	IVSQWLGRKR AMLFSNALAV LGGTLMGLAK	AAASYEM LIL GRFFIGAYSG		150	
Pig	LASGLVPMYV GEIAPTHLRG	ALGTLNQLAI VIGILIAQVL	GLESMLGTAT	200	
Human	LTSGLVPMYV GEIAPTHLRG	ALGTLNQLAI VIGILIAQVL	GLESLLGTAS	200	
Rabbit	LTSGLVPMYV GEIAPTHLRG	ALGTLNQLAI VIGILIAQVL	GLESMLGTAT	200	
Cattle	LTSGLVPMYV GEIAPTHLRG	ALGTLNQLAI VIGILIAQVL	GLESMLGTAT	200	
Pig	LWPLLIGITV LPALLQLVLL PFC	PESPRYL YIIRNLEGPA RKSLKRLTGW		250	
Human	LWPLLIGITV LPALLQLVLL PFC	PESPRYL YIIRNLEGPA RKSLKRLTGW		250	
Rabbit	LWPLLIGITV LPALLQLVLL PFC	PESPRYL YIIRNLEGPA RKSLKRLTGW		250	
Cattle	LWPLLIGITV LPALLQMVLL PLC	PESPRYL YIIRNLEGPA RKSLKRLTGW		250	
Pig	ADVSGALAEI KEEKRQLERE RPLSLLQLLG	SRTHROPLI AVVLQLSQOL		300	
Human	ADVSGVLAEL KDEKRLERE RPLSLLQLLG	SRTHROPLI AVVLQLSQOL		300	
Rabbit	ADVSGALAEI KEEKRQLERE RPLSLPQLLG	SRTHROPLI AVVLQLSQOL		300	
Cattle	ADVSEVLAEL KEEKRQLERE RSLSLLQLLG	SRTHROPLI AVVLQLSQOL		300	
Pig	SGINAVFYYS TSIF	ETAGVG QP	AYATIGAG VVNTVFTLVS VLLV	ERAGRR	350
Human	SGINAVFYYS TSIF	ETAGVG QP	AYATIGAG VVNTVFTLVS VLLV	ERAGRR	350
Rabbit	SGINAVFYYS TSIF	ETAGVG QP	AYATIGAG VVNTVFTLVS VFLV	ERAGRR	350
Cattle	SGINAVFYYS TSIF	ESAGVE KP	AYATIGAG VVNTVFTLVS VFLV	ERAGHR	350
Pig	TLHL LGLAGM CGCAILMTVA LLPL	ERVPAM SY	NSIVAIFG FVAFFEIGPG		400
Human	TLHL LGLAGM CGCAILMTVA LLL	ERVPAM SY	NSIVAIFG FVAFFEIGPG		400
Rabbit	TLHL LGLAGM CGCAILMTVA LLL	ERVPAM SY	NSIVAIFG FVAFFEIGPG		400
Cattle	TLHL LGLAGM CACAILMTVA LLL	ERVPAM SY	NSIVAIFG FVAFFEIGPG		400
Pig	PIPWFVAEL FSQGPRPAAM AVAGFSNWTS	NFIIGMGEFOY VAD	AMGPYVF	450	
Human	PIPWFVAEL FSQGPRPAAM AVAGFSNWTS	NFIIGMGEFOY VAD	AMGPYVF	450	
Rabbit	PIPWFVAEL FSQGPRPAAM AVAGFSNWT	NFIIGMGEFOY VAD	AMGPYVF	450	
Cattle	PIPWFVAEL FSQGPRPAAM AVAGFSNWT	NFIIGMGEFOY VAD	AMGPYVF	450	
Pig	LLFAVLLLG FIFTFL	KVPE TRGRITFDQIS AAFRRTPSLL	EQEVKPSSTEL	500	
Human	LLFAVLLLG FIFTFL	KVPE TRGRITFDQIS AAFRRTPSLL	EQEVKPSSTEL	500	
Rabbit	LLFAVLLLG FIFTFL	KVPE TRGRITFDQIS AAFRRTPSLL	EQEVKPSSTEL	500	
Cattle	LLFAVLLLG FIFTFL	KVPE TRGRITFDQIS AAFRRTPSLL	EQEVKPSSTEL	500	
Pig	EYLGPDEND			509	
Human	EYLGPDEND			509	
Rabbit	EYLGPDEND			509	
Cattle	EYLGPDEND			509	

Figure 3. Amino acid sequence of GLUT4 from pigs and other three mammalian species. Putative transmembrane domains are indicated by open box.

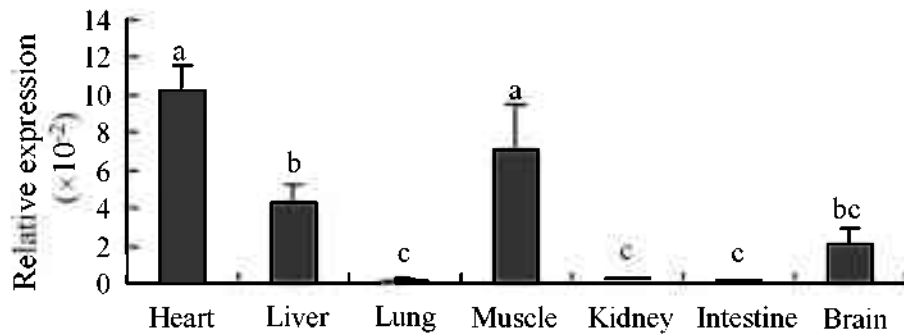


Figure 4. Tissue distribution pattern of porcine GLUT4. All samples were normalized using β -actin as an internal reference gene in Real-time RT-PCR. Different characters in bars show that there is significant difference among different tissues ($p < 0.05$).

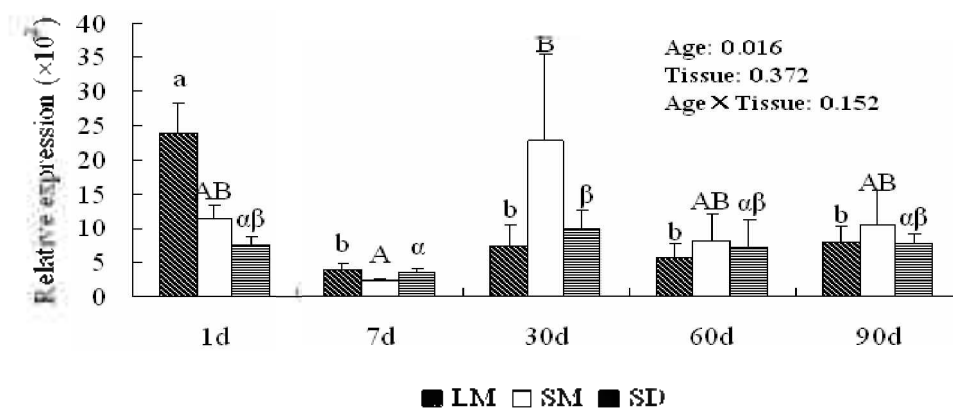


Figure 5. Relative expression of GLUT4 mRNA in skeletal muscle during Landrace development. All samples were analyzed by Real-time RT-PCR for expression of GLUT4 and β -actin. Expression levels of GLUT4 were normalized to β -actin. Units represent the relative expression of GLUT4. LM: *Longissimus dorsi* muscle; SM: *Semimembranosus* muscle; SD: *Semitendinosus* muscle. Different characters in the bars with same color mean that there is significant difference in the mRNA expression of GLUT4 in skeletal muscle from Landrace with different ages ($p < 0.05$).

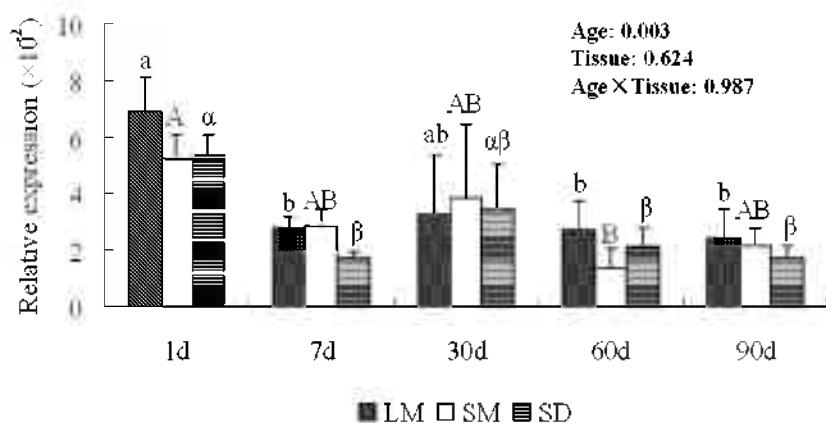


Figure 6. Relative expression of IR mRNA in skeletal muscle during Landrace development. All samples were analyzed by Real-time RT-PCR for expression of IR and β -actin. Expression levels of IR were normalized to β -actin. Units represent the relative expression of IR. LM: *Longissimus dorsi* muscle; SM: *Semimembranosus* muscle; SD: *Semitendinosus* muscle. Different characters in the bars with same color mean that there is significant difference in the mRNA expression of IR in skeletal muscle from Landrace with different ages ($p < 0.05$).

In comparison with development of GLUT4 mRNA, the insulin receptor (IR) mRNA was detectable in skeletal muscle tissue from post-natal Landrace pigs (Figure 6). There was significant difference in the relative expression of IR in skeletal muscle from Landrace pigs at different ages ($p = 0.003$). Similarly, the relative mRNA expression of IR in skeletal muscles at the age of 1 and 30 d was also higher than that at the other ages. The mRNA expression level of IR from LM and SD at the age of 1 d was significantly higher than those at the age of 7, 60 and 90 d ($p < 0.05$). And the mRNA expression level of IR from SM muscle at the age of 1 d was significantly higher than those at the age of 60 d from SM muscle ($p < 0.05$). Interestingly, there was also no significant difference in the relative expression level of IR mRNA among different skeletal muscle tissues ($p > 0.05$). Moreover, there was also no significant interaction between age and tissue for IR mRNA in this study ($p > 0.05$).

DISCUSSION

A genome wide GeneBank search indicates that the GLUT1-12 and HMIT (H+/myo-inositol transporter, GLUT13) may represent all facilitative glucose transporter members in human. Sequence analysis of these 13 family members shows that the sequences are more conserved in the putative transmembrane regions (Zhao et al., 2007). In this study, we have cloned the cDNA of porcine GLUT4. The porcine GLUT4 encodes a peptide of 509 amino acids residues that exhibits an over 90% amino acid identity to the GLUT4 of human, rabbit and cattle. Moreover, the deduced putative transmembrane regions of porcine GLUT4 were also similar to these of human, rabbit and cattle. These results indicated that the molecular identity of cDNA in this study might be porcine GLUT4.

In the present study, we have found that the porcine GLUT4 mRNA was detected in heart, liver, skeletal muscle, brain, lung, kidney and intestine. And the heart showed the highest expression level of GLUT4 among these tissues, followed by skeletal muscle, liver, brain, but with lower level in other tissues. The highest GLUT4 mRNA in heart suggested that piglets may need glucose as an energy source for heart growth. Similarly, broilers also expressed high mRNA level of GLUT1 in heart at the age of 7 d (Humphrey et al., 2004). GLUT4 expression in young porcine liver appears to differ from that in Atlantic cod, where it is highly expressed for GLUT2 mRNA (Hall et al., 2006). These reports suggested that it was very important for glucose transport in liver and it may need enough much glucose as energy source for liver's function. The absorption of free glucoses from the small intestine is believed to be a two-step process, including a passive

process and an active process with variation of sodium gradient. And the mechanisms of glucose re-absorption across the tubule in kidney are considered to be similar to these in the small intestine. It has been well reviewed that the primary glucose transporters in small intestine were SGLT1, GLUT2 and GLUT5, and SGLT2 in kidney (Zhao and Keating, 2007). In the present study, the relatively low mRNA of GLUT4 in intestine and kidney is consistent with that reported for GLUT4 in atlantic cod (Hall et al., 2006). In addition, porcine skeletal muscle tissue expressed relatively high level of GLUT4 mRNA in this study. The glucose transport activity in L6 muscle cells was regulated by the coordinate control of sub-cellular glucose transporter distribution, biosynthesis, and mRNA transcription (Walker et al., 1990). The high abundance of GLUT4 mRNA expression in skeletal muscle may suggest that the expression of this isoform plays a key role in glucose metabolism in this tissue.

The glucose transporter GLUT4 is primarily responsible for the increase in glucose uptake with responsible to insulin stimulation. Many reports have proved that insulin could stimulate glucose uptake (He et al., 2003; Hou and Pessin, 2007; Manso Filho et al., 2007). The present study showed that there was a large decrease in the relative expression level of GLUT4 and IR mRNA in porcine skeletal muscle by 1-7 d and a large increase by 7-30 d. The developmental changes in the mRNA abundance of GLUT4 coincide with these in IR. The number of insulin receptors is an important determinant of the specificity of insulin action (Kitamura et al., 2004). Thus it appears that piglets may increase glucose metabolism by increasing muscle GLUT4 mRNA and insulin sensitivity at birth and after 2 d of weaning period. However, the changes in the mRNA abundance of GLUT4 and IR are not always the same. Previous study has shown that GLUT4 mRNA was expressed at low levels at the age of 1-8 d and at 2-3 fold higher levels during and after weaning (18-40 d) in rat skeletal muscle while there was little change in IR mRNA prior to weaning and a reduction in mRNA abundance between 18 and 40 d (Knott et al., 1992). Recent data have also shown that the IR was expressed from the morula stage onwards while the insulin-sensitive isoform GLUT4 was not expressed (Tonack et al., 2009). It is likely therefore that porcine skeletal muscle might be a classic insulin target model to illustrate the influence of insulin signaling on GLUT4 by *in vivo* study.

Previous studies have shown that glucose transporter expression in muscle is regulated by several factors, including dietary factors (Le et al., 2008), hormone (Gray et al., 2006), developmental regulation (Humphrey et al., 2004), and contractile activity (Heled et al., 2005). However, there is little information to study the influence of multi-

factors interaction on muscle GLUT4 expression. A recent published data has found that the regulation of GLUT4 in brown trout took place predominantly in the red skeletal muscle by insulin treatment (Diaz et al., 2007). In the current study, no significant interaction between age and tissue was found in the mRNA expression of GLUT4 or IR, but the mRNA expression peak of GLUT4 in different skeletal muscles differed. We have previously shown that the proportion of red fibers in porcine LM muscle was different from that in SD and SM (Unpublished). Thus, it suggests that the postnatal development of porcine GLUT4 mRNA might be muscle specific. In addition, the study of GLUT1 through 5 expression in developing and mature human skeletal muscle has found that GLUT4 seemed to be of importance during muscle fiber growth development (Gaster et al., 2000). In this study, porcine skeletal muscle always kept high expression of GLUT4 mRNA after weaning, which indicated that GLUT4 might be an important glucose transporter in skeletal muscle during the adult development.

In conclusion, porcine GLUT4 had a similar molecular structure to the insulin-responsive GLUT4 of human, rabbit and cattle, with an amino acid identity of over 90%. Moreover, mRNA expression of GLUT4 in heart, skeletal muscle and liver from piglets was relatively high. In Landrace skeletal muscle, the mRNA expression level of GLUT4 coincided with those of IR, but the variation degree differed in different skeletal muscle tissues. These results illustrate that porcine GLUT4 may be an insulin-responsive glucose transporter and play an important role in the development of skeletal muscle.

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