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Molecular characterization and expression analysis of the porcine caveolin-3 gene

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Abstract

Caveolin-3 is the muscle-specific form of the caveolin protein family and plays an important role in modulating both the morphological appearance and function of caveolae. In this study, we cloned and characterized caveolin-3 from porcine muscle. The promoter of porcine caveolin-3 contained three consensus E box elements and one RORα2 monomeric binding motif. The deduced amino acid sequence of porcine caveolin-3 contains a WW domain. This gene was mapped to SSC13 q23–q24 by the SCHP and the IMpRH panel. RT-PCR analyses showed that caveolin-3 was expressed specifically in skeletal muscle and heart. And we provide the first evidence that caveolin-3 has a certain regulated expression pattern during the prenatal period of the porcine skeletal muscle development. This result suggests that the caveolin-3 gene might be a candidate gene of meat production trait and provides some information for establishing of an animal model using pig to study human caveolinopathies.

Keywords: Pig; Caveolin-3; Skeletal muscle; Differential gene expression

The caveolin family currently has three members, which are caveolin-1 (CAV1), caveolin-2 (CAV2), and caveolin-3 (CAV3). They are important in the formation of caveolar membranes as caveolin expression levels correlate very well with the morphological appearance of caveolae [1]. Caveolae are small flask-shaped plasma membrane invaginations that participate in membrane trafficking, sorting, transport, and signal transduction [2]. CAV3 is the muscle-specific form of the caveolin protein family [3]. Unlike CAV1 and CAV2's ubiquitous expression pattern, CAV3 expressed restricted in muscle cells [4]. Recent studies using caveolin-deficient mouse models showed that caveolae and caveolins play a prominent role in various human patho-biological conditions [5]. CAV3 mutations and expression alterations can result in caveolinopathies including limb girdle muscular dystrophy, rippling muscle disease, distal myopathy, and hyperCKemia [6].

The number and size of myofibers are critical for meat production and quality [7]. 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 [8]. Vistisen et al. [9] reported that CAV3 expression in type-2 fibers is significantly higher than that in type-1 fibers in human. Therefore, the skeletal muscle fiber type may be influenced by a CAV3-dependent transcriptional pathway, and the knowledge of modulation of CAV3 gene will contribute to the understanding of muscle development.

Although mouse has been used as human caveolinopathies models, the pig is considered as an important experimental animal model of human disease, because pigs and humans share similar anatomical, physiological, and pathological characteristics. The knowledge of porcine CAV3, therefore, will also contribute to the understanding and development of porcine models for human caveolinopathies prevention and treatment.

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In this study, we characterized the mRNA sequences of porcine CAV3 and cloned the porcine CAV3 promoter, investigated its expression patterns and chromosome assignments.

Materials and methods

Animal and tissue. Two pure breed, Duroc and Chinese indigenous Tongcheng pigs, were used in this study. One Duroc boar was mated with seven Duroc sows and one Tongcheng boar was mated with seven Tongcheng sows. The fetal skeletal muscles from seven ages (45, 55, 65, 75, 90, 105, and 114 days postconception (dpc)) were harvested and then snap-frozen in liquid nitrogen.

cDNA library construction. Poly(A)$^+$ RNA for library construction was isolated from a 55 dpc porcine fetus skeletal muscle from Tongcheng pig by using the Oligotex direct mRNA midi kit and oligotex buffer set kit (Qiagen Inc., Valencia, CA), a SMART™ cDNA library construction kit (Clontech, Palo Alto, USA) was used for construction the full-length cDNA library. In order to check the insert size of the clones of the cDNA library, plasmid DNA was extracted from 200 randomly selected clones with BioDev plasmid DNA purification kit (BioDev Inc., China). The *Sfi*IA and *Sfi*IB restriction enzymes were used to check the inserted fragments in the plasmids. The clones with the average inserted size of about 2 kb were then sequenced from the 5′-end with M13 reverse primer by ABI377 automated DNA sequencer. One sequence showed high similarity with human CAV3 gene.

Reverse transcript-PCR analysis of CAV3 gene expression. Total RNA isolated from the longissimus dorsi by using Trizol reagent (Gibco-BRL, Rockville, USA) following the supplier's protocol, and treated with RNase-free DNase (Fermentas, Vilnius, Lithuania), was reverse-transcribed into cDNA using Omniscript Reverse Transcriptase (Qiagen Inc., Valencia, CA) and the oligo(dT) primer. The reverse transcription was carried out in a final volume of 20 μL containing 1 μg of total RNA. The mixture was incubated for 1.5 h at 37 °C. Two microliters of the resulting single-stranded cDNA was amplified 25 cycles with CAV3-specific primers (Table 1). A specific primer pair (Table 1) that amplified the 309-bp housekeeping gene GAPDH was used as internal control. PCR products were separated by electrophoresis on 2.0% agarose gels and visualized by ethidium bromide staining. The PCR fragments were purified and directly sequenced to confirm the correct amplification of the porcine CAV3 gene.

For temporal expression analysis of CAV3, total RNAs were isolated from longissimus dorsi muscle from various fetal developmental stages (45, 55, 65, 75, 90, 105, and 114 dpc), three individual fetuses were detected in each stage in order to do ANOVA analysis. For tissue-specific expression analysis, total RNAs were isolated from various tissues (heart, liver, skeletal muscle, lung, spleen, kidney, and bladder).

SYBR Green real-time RT-PCR analysis of gene expression patterns. The expression level of CAV3 gene in three developmental stages was detected by SYBR Green I assay. Each real-time PCR (in 20 μL) reaction contained 1× PCR buffer (TaKaRa), 2.0 mM $MgCl_2$, 500 μM each dNTP, 0.4 μM primers (Table 1), 0.3× SYBR Green I, and 1 U *Taq* DNA polymerase (TaKaRa) plus 2 μL normalized template cDNA. The cycling conditions consisted of an initial, single cycle for 3 min at 95 °C followed by 35 cycles of cycling consisting of 15 s at 94 °C, 20 s at 61 °C, 20 s at 72 °C, and fluorescence acquisition at 82 °C for 1 s. The specific PCR products were confirmed by melting curve analysis. cDNAs from three fetus muscle samples in each stage were used to detect the expression changes of the target gene, and all PCRs were performed in triplicate and gene expression levels were quantified relatively to the expression of β-actin using Gene Expression Macro software (Bio-Rad, Richmond, CA, USA) by employing an optimized comparative C_t ($^{\Delta\Delta}C_t$) value method. Expression levels were considered not detectable when the C_t value of the targeted gene exceeded 35 in the sample tissue. The *t*-test was conducted to identify genes differing in expression, $p < 0.05$ was considered significant.

Cloning and analysis of porcine CAV3 promoter. To clone the 5′ flanking region of the porcine CAV3 gene, the human CAV3 gene (GenBank Accession No. 9930104) was aligned with Bos Taurus chromosome 22 genomic contig (GenBank Accession No. NW_930087) to obtain homologous sequence for designing the primers. The primers spanned the 5′ flanking region and the exon 1 (Table 1). The amplified fragment was sequenced and deposited in GenBank (GenBank Accession No. DQ415642). Promoter prediction analysis was performed using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html), CONSITE (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite), and Transcription Regulatory Element Search (http://bioportal.bic.nus.edu.sg/tres/).

Chromosomal assignment of the porcine CAV3 gene. A somatic cell hybrid panel (SCHP) [10] was used for chromosomal assignments and the radiation hybrid (IMpRH) was employed to improve the mapping resolution [11]. Pig-specific primers (Table 1) were designed in the 3′ UTR of pig caveolin-3 gene for chromosomal mapping. The PCRs were performed in a volume of 20μL of 1× PCR buffer (Promega), consisting of 20 ng of cell hybrid line DNA, 10 pmol of each primers, 100 μM of each dNTPs, 1.5 mM MgCl2, and 2.0 U *Taq* DNA Polymerase (Promega). The PCR profile was 3 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 30 s at 72 °C, and a final extension of 5 min at 72 °C. Analysis of the PCR results was performed with the software available on http://www.toulouse.inra.fr/lgc/pig/hybrid.htm [12] and the IMpRH mapping tool (http://imprh.toulouse.inra.fr/) [13] for SCHP and RH mapping, respectively. Two-point RH analysis was used for identification of linkage groups with LOD score threshold of 3.0.

Table 1
Primers used in this study

Primer name	Primer sequence (5′–3′)	Annealing temperature (°C)
CAV3 semi-RT-PCR PL	CTCTGGGGCTTCCTGTTC	52
CAV3 semi-RT-PCR PR	CATCACCTTGATGTTGCTG	
GAPDH semi-RT-PCR PL	CCTTCATTGACCTCCACTAC	52
GAPDH semi-RT-PCR PR	GTTGTCATACTTCTCATGGTTC	
CAV3 qPCR PL	CGGCGTGTGGAAGGTGAG	61
CAV3 qPCR PR	AAGGTGCGGATGCAGAGTG	
β-Actin qPCR PL	GGACTTCGAGCAGGAGATGG	61
β-Actin qPCR PR	GCACCGTGTTGGCGTAG GG	
5′ flanking region PL	AGAGCCTTACTGGTCTGTTT	62
5′ flanking region PR	CGATCTCCTTGAAGTGAATGT	
Mapping PL	GTGATGCTGCGGAAAGAA	60
Mapping PR	GGATGGGAACTGAGTGCTG	

Note: The primer pairs CAV3 semi-RT-PCR PL and PR, GAPDH semi-RT-PCR PL and PR were employed in semi-quantitative RT-PCR, the primer pairs CAV3 qPCR PL and PR, β-actin qPCR PL and PR were used in real-time RT-PCR. 5′ flanking region PL and PR were used for identifying porcine caveolin-3's flanking region and the primer pair mapping PL and PR was employed for radiation hybrid mapping.

Results

Molecular cloning and sequence analysis of porcine CAV3 gene

The 950-bp full-length porcine CAV3 cDNA (clone ID: sus_zm51; GenBank Accession No. AY731093) was identified by randomly sequencing the Chinese Tongcheng pig 55-day fetal longissimus dorsi muscle full-length cDNA library [14]. Sequence analysis showed that porcine CAV3 cDNA contains an open-reading frame of 456 nucleotides (nt), encoding a protein of 151 amino acids with a calculated molecular mass of 17.55 kDa and an isoelectric point (p*I*) of 9.4. The 89 nt 5′-UTR and 405 nt 3′-UTR containing a consensus polyadenylation signal (AATAAA) were also identified. The putative methionine initiation codon occurs in favorable sequence context for the initiation of translation with an A in the −3 position and a G in the +4 position [15]. To investigate the transcriptional regulation of porcine CAV3 expression, we cloned and sequenced the 5′ flanking region of the porcine CAV3 gene (Fig. 1). A consensus TATA box was identified at 29 bp from the 5′ transcriptional start site. In a computer homology search for other *cis*-acting regulatory elements that have been implicated in skeletal muscle gene regulation, we identified three consensus E box elements that represent putative binding sites for basic helix-loop-helix transcription factors and one RORα2 element that functions as a constitutive *trans*-activator of gene expression (Fig. 1). Alignment with the 5′ flanking region between porcine CAV3 and human CAV3 gene showed that there were 419 bp in the 5′ flanking region of porcine CAV3 which had no homologous fragment in the human CAV3 (Fig. 1, underlined).

A GenBank database search using BLAST revealed that the predicted porcine CAV3 amino acid sequence has high similarity with other mammalian CAV3 protein sequences, with a 92% identity to human, an 88% identity to rat and mouse, respectively. The phylogenic relationship among all the hitherto characterized members of the caveolin family was illustrated according to the phylogenetic distance calculated by the CLUSTALW1.83 program [16] (Fig. 2). CAV2 first diverged into a cluster, then CAV1 and CAV3 form two separate clusters. It is generally accepted that CAV2 is expressed in a variety of cell types [17].

The transmembrane helix in porcine CAV3 predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) showed that 1–78 amino acid residues are inside, 79–101 amino acid residues are transmembrane helix, while 102–151 amino acid residues are outside [18]. This is in consistent with the Kyte–Doolittle plot of the hydrophilicity, with 74-hydrophilic N-terminal domain, a 33-amino acid membrane-spanning segment, and a 44-amino acid hydrophilic C-terminal domain. We searched for putative WW domains within the caveolin-3 protein sequence. WW

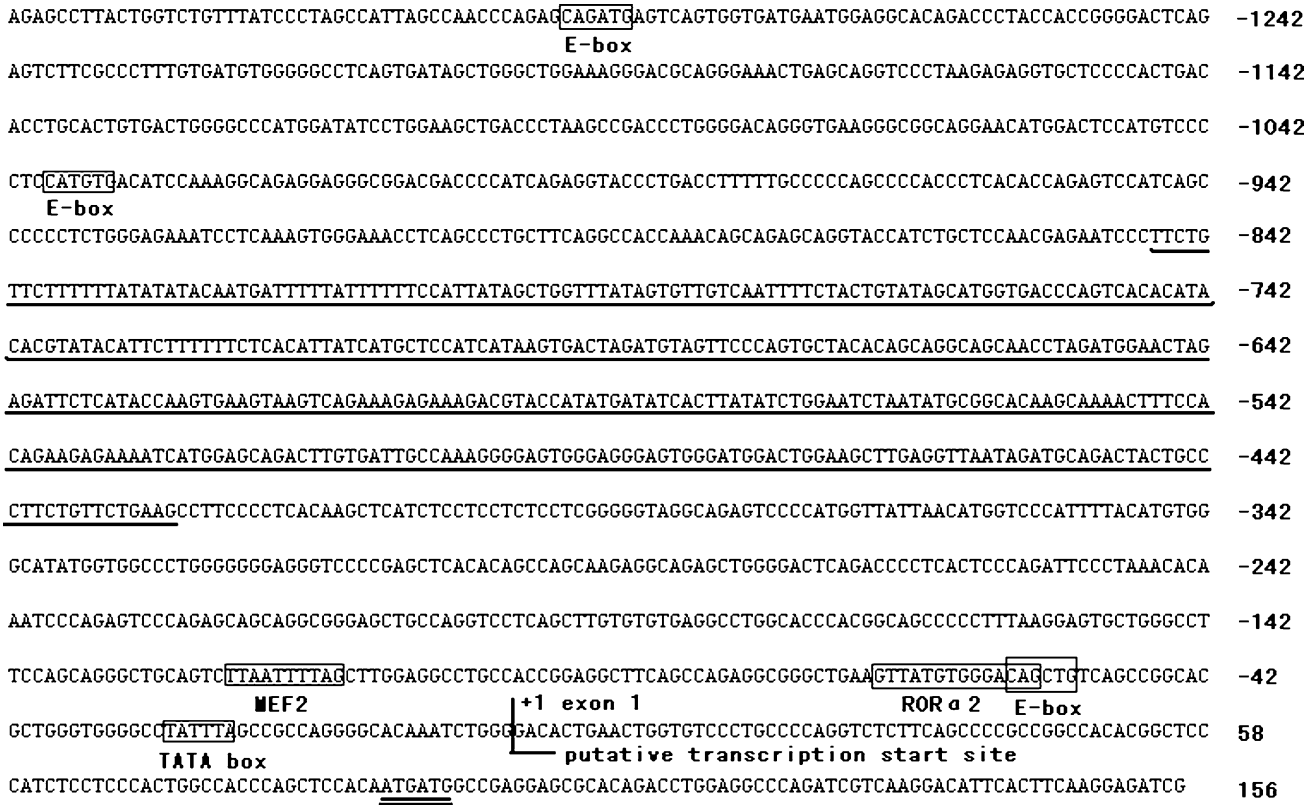

Fig. 1. The porcine CAV3 promoter sequence. Putative transcription factor-binding sites and putative TATA box are boxed. Transcriptional start site is indicated. The translation initiation site, ATG, is double underlined. The 419 bp fragment in the 5′ flanking region of porcine CAV3 which had no homologous fragment in the human CAV3 is underlined.

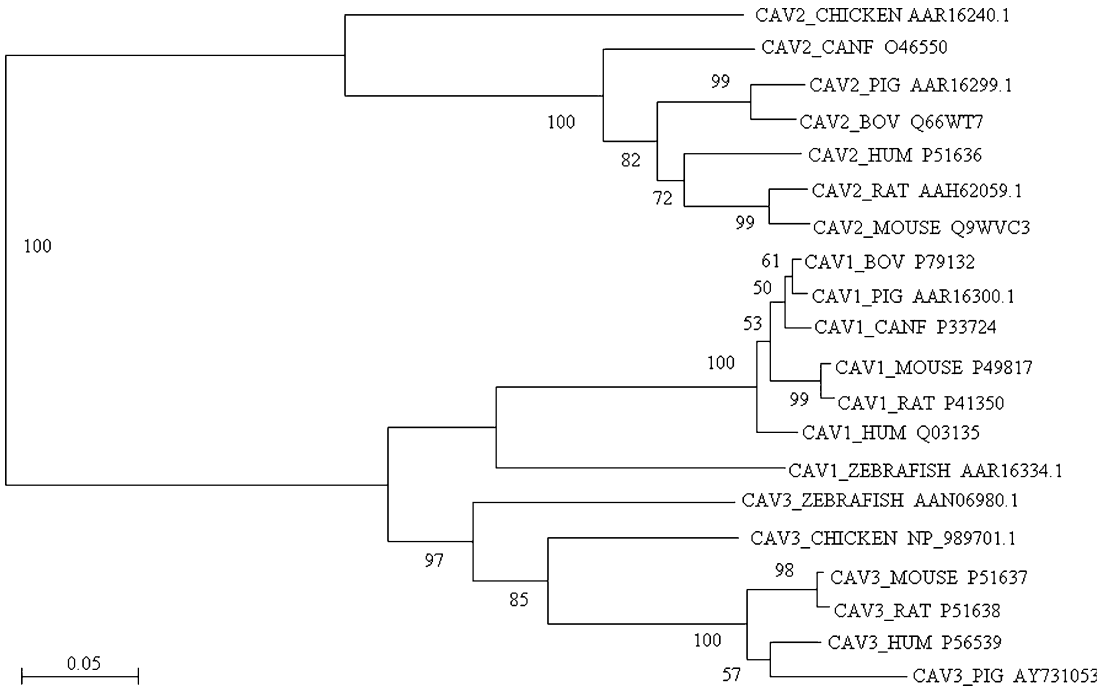

Fig. 2. Phylogenetic relationship of caveolin family members. The amino acid sequences were derived from GenBank. The bootstrap confidence values shown at the nodes of the tree were based on 8000 bootstrap replicates. The horizontal branch lengths are proportional to the estimated divergence of the sequence from the branch point.

domain is characterized as containing the conserved aromatic residues Tryptophan (W, Trp), Phenylalanine (F, Phe), or Tyrosine (Y, Tyr), and Proline (P, Pro). The two W residues separated by 29 amino acids are highly conserved among caveolin family members. There is also a highly conserved residue followed with the second tryptophan. These conserved amino acid residues are indicated with an asterisk in Fig. 3.

Chromosomal location of the porcine CAV3 gene

By PCR analysis of DNA from a somatic cell hybrid panel and a INRA-University of Mininesota porcine radiation hybrid (IMpRH) panel, the porcine CAV3 was mapped to SSC13 q23–(1/2) q24 with an error risk less than 0.5%. The closest marker is SW960 with LOD score of 3.47. The conserved syntenic region in human is on chromosome 3p25, where the human CAV3 is located.

Temporal expression of CAV3 gene during skeletal muscle development

To detect the expression pattern of CAV3 gene in porcine skeletal muscle, we performed the semi-quantitative RT-PCR analyses of seven developmental time points, and samples from three individual fetuses were detected,

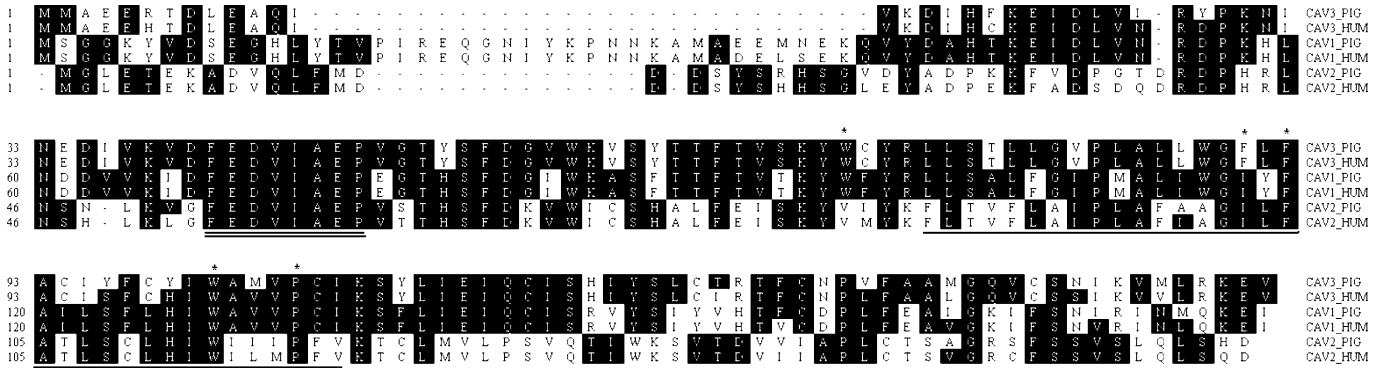

Fig. 3. Multiple alignment of human/porcine caveolin-1, -2, and -3. The deduced amino acid sequence of the porcine CAV3 was aligned to that from porcine CAV1 (GenBank Accession No. AAR16300.1), porcine CAV2 (GenBank Accession No. AAR16299.1), human CAV3 (GenBank Accession No. p56539), human CAV1 (GenBank Accession No. Q03135), and human CAV2 (GenBank Accession No. p51636). Identical amino acid residues among species are presented with a black background; the caveolin signature sequence (FEDVIAEP) are double underlined, the transmembrane helix are underlined, and the conserved amino acid residues of WW domain are indicated with asterisks.

respectively, in each stage. These analyses showed that CAV3 expression level was increased from the 45 dpc to the 75 dpc fetal muscle, whereas the expression level was down-regulated from the 75 dpc to the 114 dpc fetal muscle of both Duroc and Tongcheng pig (Fig. 4a). Moreover, pig CAV3 was significantly differentially expressed among these stages in both Tongcheng pig ($p = 9.47e{-}05 < 0.01$) and Duroc ($p = 5.84e{-}05 < 0.01$). In Tongcheng pig fetal muscle, the mRNA abundance of CAV3 in the 75 dpc was 1.75-fold of that in the 114 dpc, and in Duroc fetal muscle, the mRNA abundance of CAV3 in the 75 dpc was 1.96-fold of that in the 114 dpc. The real-time RT-PCR results showed similar expression trend with the semi-quantitative RT-PCR analyses at the corresponding three stages (Fig. 4a and b). And pig CAV3 was also significantly differentially expressed among these stages in both Tongcheng pig ($p = 3.67e{-}014 < 0.01$) and Duroc ($p = 5.84e{-}012 < 0.01$). At the 90 dpc, the real-time RT-PCR results showed that the expression level of CAV3 gene of Tongcheng pig was significantly lower than that in the Duroc ($p = 0.0016 < 0.01$) (Fig. 4b).

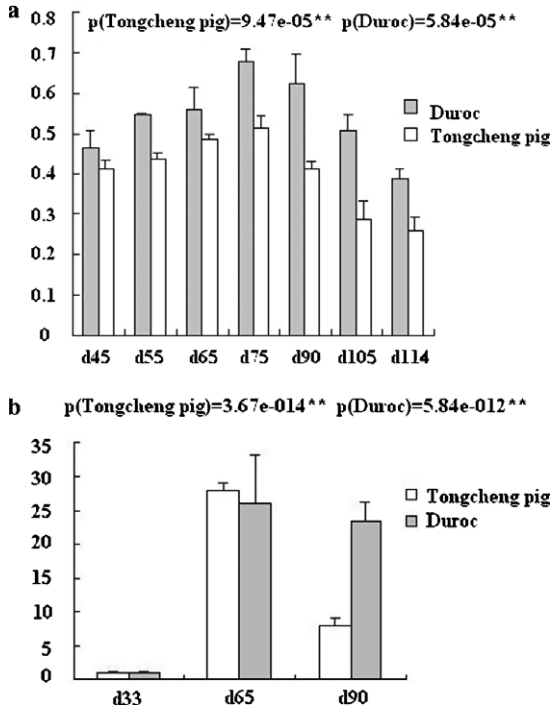

Fig. 4. The temporal distribution of porcine caveolin-3 mRNA in the fetal skeletal muscle from several different stages. (a) Semi-quantitative RT-PCR results. Error bars indicate the SD ($n = 3$) of relative mRNA expression levels of CAV3 to GAPDH. The values were normalized to the GAPDH (housekeeping gene) expression. Pig CAV3 was significantly differentially expressed among the stages in both Tongcheng pig ($p = 9.47e{-}05 < 0.01$) and Duroc ($p = 5.84e{-}05 < 0.01$). d45, 45 dpc; d55, 55 dpc; d65, 65 dpc; d75, 75 dpc; d90, 90 dpc; d105, 105 dpc; d114, 114 dpc. (b) Real-time RT-PCR results. Error bars indicate the SD ($n = 3$) of relative mRNA expression levels of CAV3 to β-actin. The values were normalized to endogenous β-actin expression. And pig CAV3 was also significantly differentially expressed among the stages in both Tongcheng pig ($p = 3.67e{-}014 < 0.01$) and Duroc ($p = 5.84e{-}012 < 0.01$). d33, 33 dpc; d65, 65 dpc; d90, 90 dpc.

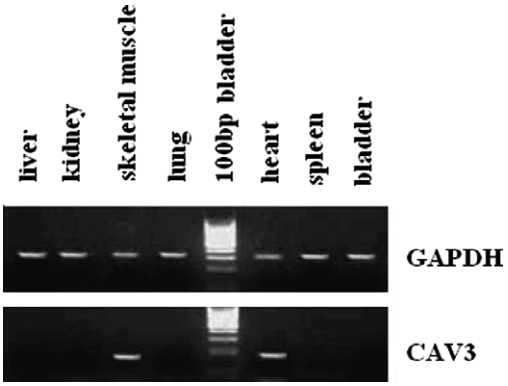

Fig. 5. Porcine CAV3 gene expression in different tissues by RT-PCR.

Tissue-specific expression of porcine CAV3 gene

Tissue expression patterns of porcine CAV3 gene were also analyzed using RT-PCR. Strong expression of CAV3 was observed in skeletal muscle and heart, and expression of porcine CAV3 was undetectable in liver, kidney, lung, spleen, and bladder (Fig. 5). Our result indicated that porcine CAV3 strictly expressed in striated muscle tissues.

Discussion

CAV3 gene has been so far characterized in human [19], mouse [20], chicken [20], and zebrafish [21]. In this study, we have obtained the full-length porcine CAV3 cDNA from the pig 55 dpc fetal longissimus dorsi muscle full-length cDNA library and cloned the porcine caveolin-3 promoter. We also mapped this gene to SSC13 q23–(1/2) q24. When browsing QTLs in the latest released Pig QTL Database (http://www.animalgenome.org/cgi-bin/QTLdb/browse) [22], several QTLs, such as longissimus muscle area, shear force, backfat weight, feed intake, body weight at birth, and average daily gain, were mapped to this small chromosomal region.

In our study, porcine CAV3 was found strictly expressed in striated muscle tissues. The result is generally in agreement with the report that CAV3 is the muscle-specific form of the caveolin protein family [4]. We identified three consensus E box elements and one RORα2 element in the 5′ flanking region of the porcine CAV3 gene, and we found that there were 419 bp in the 5′ flanking region of porcine CAV3 which had no homologous fragment in the human CAV3 promoter region. Biederer et al. [23] reported that basic helix-loop-helix transcription factors mediated specific induction of CAV3 gene expression during embryonic development. And Lau et al. [24] uncovered that RORα regulated the expression of CAV3 in skeletal muscle cells. The difference of 5′ flanking region of CAV3 in porcine may affect its expression and requires further investigation.

Our study also showed that during the porcine muscle fiber hyperplasia period, the expression of CAV3 was up-regulated, while in the period of the muscle fiber

hypertrophy stages, the expression of CAV3 was down-regulated. It was generally agreed that pig skeletal muscle fiber number stopped increasing from 65 dpc to 85 dpc, and after that the pig skeletal muscle fiber began to hypertrophy [7]. This result indicates that porcine CAV3 may have a relationship with muscle fiber development.

We also showed that the expression of CAV3 in Duroc was higher than that in Tongcheng pig. CAV3 expression is significantly higher in type-2 fibers than that in type-1 fibers in human [9]. There are more type-1 fibers in Chinese indigenous pigs than in the introduced pigs such as Duroc and Large White [25]. Gueguen et al. [26] showed that the number of primary fibers did not differ significantly between breeds, whereas the secondary/primary ratio was dramatically reduced in Meishan pig. Duroc is well known for its high growth rate and lean meat percentage, whereas Tongcheng pig has low growth rate and low lean meat percentage, but it has good meat flavor. Thus, the expression profile of porcine CAV3 indicated that CAV3 gene could have some relationship with meat production which is affected by muscle fiber type.

In summary, we have isolated and characterized the porcine CAV3 gene. Data presented in our study provide an expression and structural basis for future studies on porcine CAV3 function and will potentially lead to a better understanding of the mechanism of caveolae signaling in skeletal muscle. The expression differences in pig breeds with different muscle fiber types indicate a possible relationship to meat production. Our data also contribute to the understanding and development of porcine models for human caveolinopathies prevention and treatment.

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