Cloning, sequence characterization, and expression patterns of members of the porcine TSSK family

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ABSTRACT. Testis-specific serine kinases (TSSKs) are a family of serine/threonine kinases highly expressed in the testes that are responsible for regulating many spermatogenesis-related protein activities. Mutations in this family have a positive relationship with oligospermia and azoospermia in human and mouse. Here, five members of the TSSK family from a Banna mini-pig inbred line (BMI) were cloned, sequenced, and characterized. The

full-length coding sequences of BMI *TSSKs* varied from 807 (*TSSK3*) to 1095 bp (*TSSK1*) and encoded 268 to 364 amino acids with molecular weights in the range 30.11 to 41.34 kDa. Following comparison with *TSSK4* genes in other species, BMI *TSSK4* was found to contain three alternatively spliced variants, inform1, inform 3, and inform 4. BMI *TSSK1* and *TSSK2* are co-localized on the *Sus scrofa* chromosome (SSC) 14, and consist of a single exon; *TSSK3*, *TSSK4*, and *TSSK6* are on SSC6, SSC7, and SSC2, and consist of two, four, and one exon, respectively. Multiple protein sequence alignment and phylogenetic analysis showed that the regions spanning the S_TKc domains were more conserved between pig and other animals: with TSSK1 and TSSK2 and TSSK3 and TSSK6 displaying the greatest degree of homology across species, and the TSSK4 protein clearly distinct from other members. Multi-tissue RT-PCR showed BMI *TSSK1*, *TSSK3*, and *TSSK4* were only expressed in the testes and seminal vesicle, *TSSK2* was confined to testes only, while *TSSK6* was expressed widely in adult tissues but was highest in the testes.

Key words: Banna mini-pig inbred line; Splice isoform; Testis-specific serine kinase; Expression pattern; Gene structure; Phylogenetic analysis

INTRODUCTION

Spermatogenesis is a complicated and well-organized process through which spermatozoa are produced from male primordial germ cells. It is the male version of gametogenesis and is essential for sexual reproduction (Lindsley and Tokuyasu, 1980; Fuller, 1993). This process requires strict gene expression and involves numerous gene products, but the molecular mechanism remains poorly understood. Protein phosphorylation is the most common posttranslational protein modification in eukaryotes and it controls nearly all the cellular processes, including cell growth, cell differentiation, cell death, cell metabolism, etc. (Manning et al., 2002; Caenepeel et al., 2004; Johnson, 2009). Considering the integral role of protein phosphorylation, it is not surprising that it is involved in spermatogenesis. As a protein kinase family, the testis-specific serine kinases (TSSKs) are noteworthy because they are testis specific (Bielke et al., 1994; Kueng et al., 1997; Zuercher et al., 2000; Hao et al., 2004; Chen et al., 2005; Wei et al., 2007).

Bioinformatics analysis has revealed that TSSKs belong to the 5'-adenosine monophosphate-activated protein kinase (AMPK) branch on the human kinome tree and they have the same conserved domain, serine/threonine protein kinase, a catalytic domain (S_TKc). The S_TKc domain is helpful in forming the precise spatial arrangement required to catalyze the transfer of γ-phosphate from ATP to the hydroxyl group of serine, threonine, or tyrosine of the target protein (Kueng et al., 1997; Chen et al., 2005; Wei et al., 2007). To date, five members of the TSSK family, including TSSK1, TSSK2, TSSK3, TSSK4, and TSSK6 have been identified in mouse and human (Kueng et al., 1997; Zuercher et al., 2000; Visconti et al., 2001; Hao et al., 2004; Bucko-Justyna et al., 2005; Chen et al., 2005; Spiridonov et al., 2005; Gnanaraj et al., 2013). Of these, TSSK1 and TSSK2 are the most well studied, and have been found to be intron-less and limited to the stage of late spermatid to sperm development in the testis of sexually mature males (Li et al., 2011).

Research on association of *TSSK2* mutations with human idiopathic infertility has shown that allele G of c.80A>G (rs3747052) and allele T of c.774C>T (rs1052756) may be the risk factors for the development of spermatogenic impairment (Zhang et al., 2010). Almost concurrently, two research groups cloned mouse and human *TSSK3* (Zuercher et al., 2000; Visconti et al., 2001); however, there is controversy about mouse TSSK3 protein expression. The original study found it was present exclusively in testicular Leydig cells (Zuercher et al., 2000), but later Li et al. (2011) were unable to detect it either in testis extracts or in mature sperm. Human TSSK4 has been found to be exclusively expressed in the testis and is located in the acrosome of the spermatid. *In vitro* experiments have demonstrated that human TSSK4 can phosphorylate the transcription factor cAMP responsive element binding protein (CREB), and stimulate the CREB/CRE responsive pathway, which suggests that CREB is likely to be one of the important substrates of human TSSK4 (Chen et al., 2005). TSSK6, also named small serine/threonine kinase, is expressed in the elongating spermatids, can phosphorylate histones, and is associated with heat shock proteins HSP90 and HSC70 (Spiridonov et al., 2005; Jha et al., 2013). Further association study in humans revealed that allele T may be a risk factor for male infertility, while alleles C and G may decrease susceptibility to male infertility in c.822+126T>G/C of TSSK6 (Su et al., 2010).

The above evidence strongly suggests that TSSKs are essential for spermatogenesis and male fertility. Although a large number of TSSK genes have been found to date, such data for swine are not available. The Banna mini-pig inbred line (BMI) is the world's first successful cultivation of a large mammal inbred line, which was exploited by Yunnan Agricultural University from the 1980s based on small-ear pigs at Xishuangbanna, Yunnan Province. As the heterozygotic genes were separated and recombined in the process of inbreeding, BMI has already owned six families and eighteen substrains with different phenotypes and genotypes and the inbreeding coefficient is up to 99.95%. Due to its physiological and anatomical similarities to human and good repetitiveness, BMI is a promising animal model for biological study (including male infertility) (Zeng and Zeng, 2005; Wang et al., 2012; Wei et al., 2013). With the aim of a detailed investigation of the TSSK family in porcine, this study isolated the full-length coding sequences (CDS) of BMI TSSKs, conducted sequence analysis, and presented their expression profiles in different tissues and during different developmental stages. The data will provide a primary foundation for further research on these porcine genes and for understanding the role of the TSSK family in BMI boars' fertility.

MATERIAL AND METHODS

Pigs and collection of RNA samples

All animal experimental procedures were approved by the Animal Care Committee of Yunnan Agricultural University, China. The pigs were raised in an insulated but unheated shed with a half slatted floor at the Banna mini-inbred line farm at Yunnan Agricultural University.

Thirty-five types of tissue samples, including the cerebrum, cerebellum, hypothalamus, brainstem, spinal cord, pituitary, heart, liver, spleen, lung, kidney, skin, muscle, duodenum, jejunum, ileum, colon, cecum, rectum, stomach, pancreas, esophagus, lymph node, testes, epididymis, submaxillary gland, thyroid, adrenal glands, sublingual gland, thymus, conarium, bulbourethral gland, seminal vesicle, prostate gland, and bladder were collected from a 250-day old BMI boar immediately after slaughter. All of above tissues were snap-frozen in liquid nitrogen and stored at -80°C before use.

RNA isolation and cDNA cloning of porcine *TSSK* genes

Total RNA was extracted from the 35 sampled tissues using the RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer protocol. Digestion with RNase-free DNase I (TaKaRa) removed genomic DNA contamination before continuing with first-strand cDNA synthesis. Reverse transcription was carried out using 3 µg RNA, which was reverse transcribed with an oligo (dT) 18 primer and M-MLV reverse transcriptase (Invitrogen, USA) following the manufacturer protocol.

Primers were designed according to pig *TSSK2-6* which were predicted by computational approaches and cattle *TSSK1* using the Primer Premier 5.0 software (http://www.premierbiosoft.com/). The primer pairs are listed in Table 1. The pooled cDNA from the above 35 tissues was used as a template to isolate BMI TSSKs. PCR was carried out in a 25 µL reaction volume containing 2.0 µL cDNA (50 ng/µL), 2.0 µL 2.5 mM mixed deoxyribonucleotide triphosphates (dNTPs), 12.5 µL 2X GC buffer I (TaKaRa), 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer, 0.25 µL 5 U/L Ex Taq DNA polymerase (TaKaRa), and 7.25 µL sterile water. The PCR cycling conditions were as follows: 98°C for 1.5 min, 35 cycles of 94°C for 30 s, 57.8 to 64.6°C for 45 s (Table 1), and 72°C for 1 min, with a final step at 72°C for 10 min. The PCR products were then purified, inserted into a pMD-18T vector, and sequenced bi-directionally using a commercial fluorometric method. At least five independent clones were sequenced for each gene.

Table 1. RT-PCR primers used to amplify Banna mini-pig inbred line testis-specific serine kinase (*TSSKs*) genes.

Primer name	Sequence (5' to 3')	Product length (bp)	Annealing temperature (°C)	Reference sequence
TSSK1-F1	ATGGATGACGCTGCCGTCCTCAAG	1104	61.5	NM_001083710.2 (cattle)
TSSK1-R1	CTAAGTGTGTGTCTCTGAGGGCTG			
TSSK2-F1	CGCTCCTGGCACCATGGAT	1094	63.2	XM_001929583
TSSK2-R1	ACCGCTAGGTGCTTGCCTTCC			
TSSK3-F1	CAGACGGAGAATGCTTTAGCCC	975	57.8	XM_003356282
TSSK3-R1	GACTTGCCATTGCTTTTATCAAGTG			
TSSK4-F1	CCAGCATCCCAAACTTGTGT	1156/1147/1129	57.8	XM_003128547
TSSK4-R1	CTCCCTCAGCCACTTTCAGGT			
TSSK6-F1	CCCCGACAGCAGCCGTCTCC	1039	64.6	XM_003123571
TSSK6-R1	TTCCGAACAAGGGCCACCGAG			

Bioinformatic analysis

The theoretical isoelectric point and molecular weight of all TSSK proteins were predicted using the Compute pI/Mw Tool on the ExPASY sever (http://us.expasy.org/tools/pi_tool.html) (Wilkins et al., 1999). Intron/exon structures were analyzed using the online SIM4 program (http://pbil.univ-lyon1.fr/sim4.php) (Florea et al., 1998) and GSDS (http://gsds.cbi.pku.edu.cn) (Guo et al., 2007) by comparing the CDS with their genomic sequences. The protein-conserved domain was analyzed using the online simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). The BLASTp program was used to search for similar proteins (http://www.ncbi.nlm.nih.gov/Blast). CLUSTAL X 2.0 (Larkin et al., 2007) was used to align amino acid sequences using the standard settings, and MEGA version 5.2 was used to construct phylogenetic trees using the neighbor-joining method with a bootstrap test of 10,000 replicates (Kumar et al., 2008).

Expression patterns of porcine *TSSK* genes

We conducted semi-quantitative RT-PCR to detect the expression levels of porcine

TSSK genes in 35 tissues. Porcine 18S rRNA (NR_046261), a reference gene, was used as an internal control as previously described (Huo et al., 2012). The control primers used were 5'-AATTCCGATAACGAACGAGAC-3' and 5'-GGACATCTAAGGGCATCACAG-3', with an amplicon size of 145 bp. The primers used to perform semi-quantitative RT-PCR for tissue expression profile analysis were the same as those described above (Table 1).

RESULTS

Sequence characteristics of porcine *TSSK* cDNAs and proteins

After RT-PCR, the entire coding regions of the porcine *TSSK* genes were obtained. Following comparison with *T*SSK4 genes of other species, porcine *TSSK4* was found to contain 3 alternatively spliced variants, *pTSSK4-1*, *pTSSK4-3*, and *pTSSK4-4*. The sequences of *pTSSK4-1*, *pTSSK4-3*, and *pTSSK4-4* were submitted to the GenBank database under accession Nos. KF640090, KF640089, and KF640088, respectively (Table 2). Other sequences of the porcine *TSSK* family were also deposited into the GenBank database under accession Nos. F640093 (*TSSK1*), KF640092 (*TSSK2*), KF640091 (*TSSK3*), and KF640087 (*TSSK6*) (Table 2).

Table 2. Sequence characteristics of Banna mini-pig inbred line testis-specific serine kinase (*TSSKs*) genes.

Gene name	Accession No.	cDNA (bp)	ORF (bp)	Protein (aa)	Mw (kDa)	pI
TSSK1	KF640093	1104	1095	364	41.34	6.53
TSSK2	KF640092	1094	1077	358	40.94	9.18
TSSK3	KF640091	975	807	268	30.11	6.18
TSSK4 infrom1	KF640090	1156	987	328	37.42	8.91
TSSK4 inform3	KF640089	1247	987	328	36.43	8.78
TSSK4 infrom4	KF640088	1229	969	322	35.87	8.66
TSSK6	KF640087	1039	822	273	30.21	9.30

ORF = open reading frame; Protein = number of amino acids in protein; Mw = molecular weight; pI = isoelectric point.

Chromosomal location and gene structure of porcine *TSSK* genes

The five identified porcine *TSSK* sequences were mapped to the pig genome assembly (Sscrofa10.2) using BLAST to determine their location. They were localized to 4 *Sus scrofa* chromosomes (SSC14, SSC6, SSC7, and SSC2). By comparing available gene annotations for human in the NCBI database with our mapping results, the TSSK-containing syntenic regions for the two species were identified with the help of evolutionarily conserved flanking markers around the TSSK genes, such as DGCR2 and DGCR14 for SSC14, EIF3I and YARS for SSC6, IPO4 and TINF2 for SSC7, and GATAD2A and CILP2 for SSC2 (Figure 1). We found that the location of *TSSK1* differs between human and pig. BMI *TSSK1* is on SSC14 while human TSSK1 is mapped to two chromosomes (Figure 2), one is on *Homo sapiens* chromosome (HSA) 22, which is designated as *TSSK1a*, the other is on HSA5, which is designated as *TSSK1b*. The *TSSK1a* is a truncated, non-transcribed pseudogene, while *TSSK1b* is the human homologue of BMI *TSSK*1 (Hao et al., 2004). In addition, the gene order of *TSSK4* between human and pig differed slightly (Figure 1).

Subsequently, the exon/intron structures of porcine *TSSK* genes were obtained using the online SIM4 and GSDS software with cDNA and genomic sequences. Results showed that porcine *TSSK1*, *TSSK2*, and *TSSK6* are intron-less, *TSSK3* and *4* have two and four exons, respectively,

which are consistent with those of human (Hao et al., 2004). As for three variants of porcine *TSSK4*, they are attributed to alternative splicing. The fourth exon of infrom1 initiated downstream of the other variants, while the third exon of infrom3 initiated upstream of the other variants. All exon-intron splice junction sequences conform to the GT-AG rule (Figure 2).

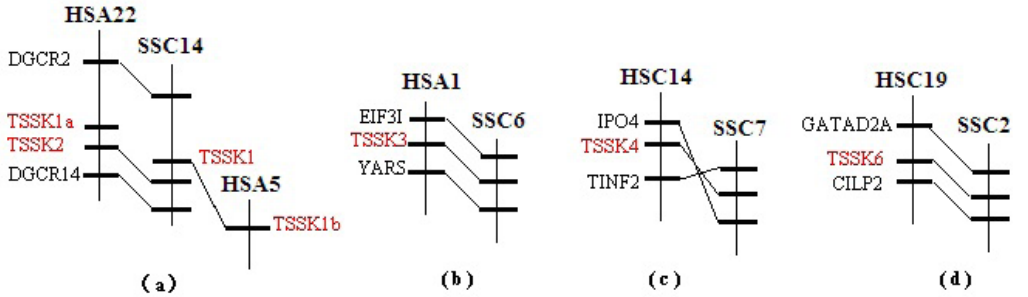

Figure 1. Comparison of testis-specific serine kinase (TSSK)-containing chromosomal regions between human and pig genomes. *TSSK* genes and their evolutionarily conserved flanking markers are shown. Genes with orthologous relationships are indicated by lines between maps of different species. Information from the human genome build 37.2 and Sscrofa10.2 were used for humans and pigs, respectively. DGCR: DiGeorge syndrome critical region gene; EIF3I: Eukaryotic translation initiation factor 3, subunit I; YARS: Tyrosyl-tRNA synthetase, cytoplasmic; IPO4: Importin 4; TINF2: TERF1 (TRF1)-interacting nuclear factor 2; GATAD2A: GATA zinc finger domain containing 2A; CILP2: CAP-Gly domain-containing linker protein 2.

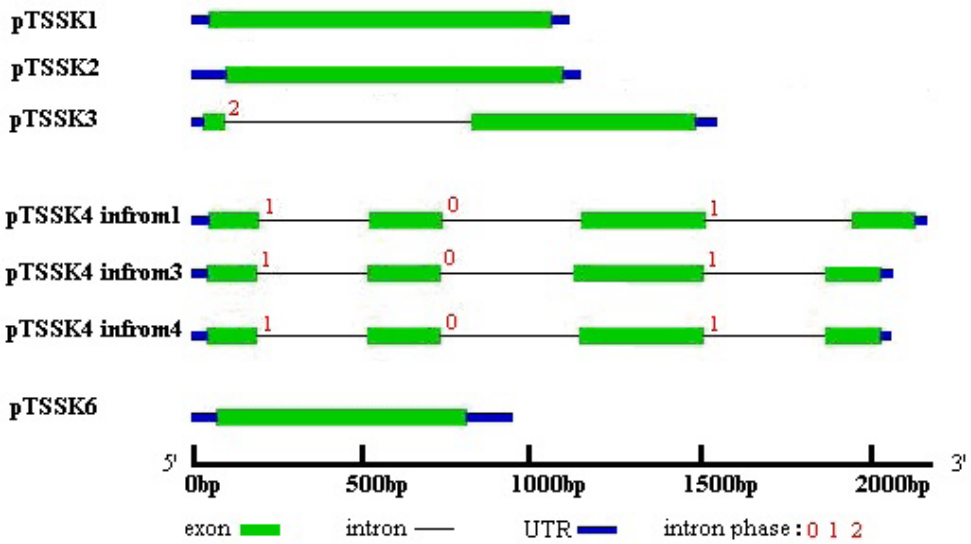

Figure 2. Schematic representation of the gene structures of Banna mini-pig inbred line testis-specific serine kinases (*TSSKs*). Exons, UTRs, introns, and intron phases are shown.

Conserved domains and evolutionary relationships of TSSK proteins

A search for conserved domains in the seven porcine TSSK proteins using the SMART domain database revealed that all contained serine/threonine protein kinases, a catalytic domain

(S_TKc; SMART No.: SM00220; Figure 3). The S_TKc domain functions by helping to form the precise spatial arrangement during the phosphotransfer process, and human and mouse TSSK proteins also have this domain (Wei et al., 2007).

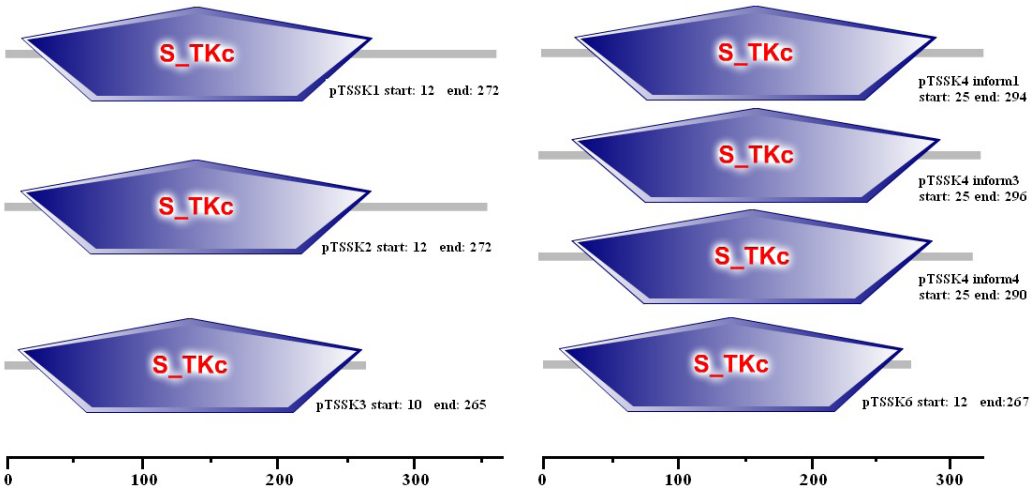

Figure 3. Predicted functional domain of Banna mini-pig inbred line testis-specific serine kinase (TSSK) proteins using SMART. S_TKc denotes serine/threonine protein kinases, a catalytic domain.

Multiple alignment for whole amino acids was performed between the porcine TSSK proteins and their orthologs in three other species (human, cattle, and mouse; Figure 4). The multiple sequence alignment results indicate that the region across the S_TKc domain is predictably highly conserved, while the N- and C-terminals have relatively low sequence identity, and porcine TSSK4-3, human TSSK4-1, and mouse TSSK4-3 and 4 have an insertion of amino acid residues in region VIb compared with other TSSK4 proteins (Figure 4). The twelve functional regions of protein kinases were assigned according to Hanks and Hunter (1995).

The cited sequences are NP_001077179.1 (cTSSK1), NP_114417.1 (hTSSK1), NP_033461.2 (mTSSK1), KF640093 (pTSSK1), XP_005195214.1 (cTSSK2), NP_443732.3 (hTSSK2), NP_033462.2 (mTSSK2), KF640092 (pTSSK2), NP_001193754.1 (cTSSK3), NP_443073.1 (hTSSK3), NP_536690.1 (mTSSK3), KF640091 (pTSSK3), NP_001178274.1 (cTSSK4), NP_001171668.1 (hTSSK4-1), NP_777004.2 (hTSSK4-2), NP_081949.1 (mTSSK4-1), NP_001240817.1 (mTSSK4-3), NP_001240818.1 (mTSSK4-4), KF640090 (pTSSK4-1), KF640089 (pTSSK4-3), KF640088 (pTSSK4-4), XP_588888.3 (cTSSK4), NP_114426.1 (hTSSK4), NP_114393.1 (mTSSK4), and KF640087 (pTSSK4).

Subsequently, the evolutionary relationships of TSSK proteins in the four species were investigated using phylogeny tree analysis (Figure 5), which showed the relationships among the TSSK family proteins more clearly. The tree divided the TSSK proteins into three large branches with at least 50% bootstrap support and revealed TSSK1 and 2, and TSSK3 and 6 across species shared the greatest degree of homology because they clustered together into one large group, while TSSK4 was very divergent from the other TSSK members.

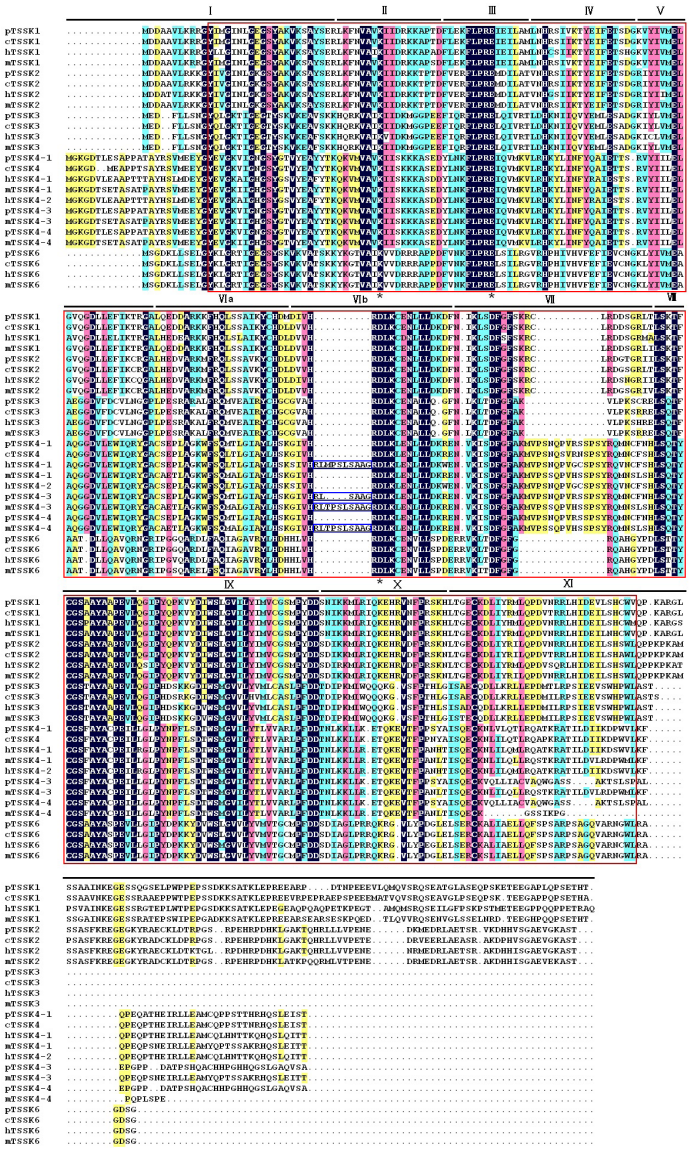

Figure 4. Alignments of testis-specific serine kinase (TSSK) proteins among human, cattle, mouse, and pig. Identical residues are represented in black and similar residues in gray. Roman numerals denote the different functional kinase domains, according to Hanks (Hanks and Hunter, 1995). The potential S_TKc domain is boxed in red and the represented amino acid residue insertion in region, VIb, is boxed in blue. Asterisks denote the indispensable residues of lysine, glutamine, and aspartic acid in the S_TKc domain. The cited sequences are NP_001077179.1 (cTSSK1), NP_114417.1 (hTSSK1), NP_033461.2 (mTSSK1), KF640093 (pTSSK1), XP_005195214.1 (cTSSK2), NP_443732.3 (hTSSK2), NP_033462.2 (mTSSK2), KF640092 (pTSSK2), NP_001193754.1 (cTSSK3), NP_443073.1 (hTSSK3), NP_536690.1 (mTSSK3), KF640091 (pTSSK3), NP_001178274.1 (cTSSK4), NP_001171668.1 (hTSSK4-1), NP_777004.2 (hTSSK4-2), NP_081949.1 (mTSSK4-1), NP_001240817.1 (mTSSK4-3), NP_001240818.1 (mTSSK4-4), KF640090 (pTSSK4-1), KF640089 (pTSSK4-3), KF640088 (pTSSK4-4), XP_588888.3 (cTSSK4), NP_114426.1 (hTSSK4), NP_114393.1 (mTSSK4), and KF640087 (pTSSK4).

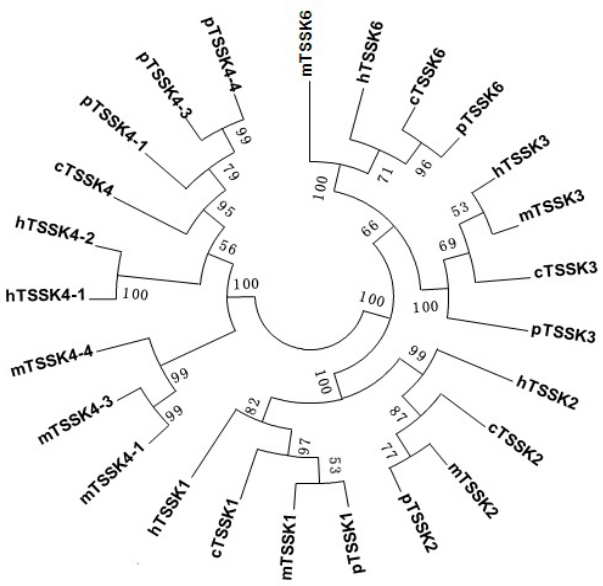

Figure 5. Phylogenetic tree based on testis-specific serine kinases TSSK protein sequences from human, cattle, mouse, and pig. The numbers on the branches represent bootstrap values for 10,000 replications.

Tissue expression patterns of porcine *TSSK* genes

The expression patterns of the porcine *TSSK* genes were studied using the semi-quantitative RT-PCR method. *TSSK2* was only expressed in the testes, implying that *TSSK2* evolved selectively to support normal testicular function. *TSSK1*, *TSSK3*, and *TSSK4* were identified only in the testes and seminal vesicle. However, porcine *TSSK6* mRNA was expressed widely: strongly in the testis, moderately in the thyroid, cecum and epididymis, weakly in the lung, spleen, and seminal vesicle, and almost silent in the other 24 tissues (Figure 6).

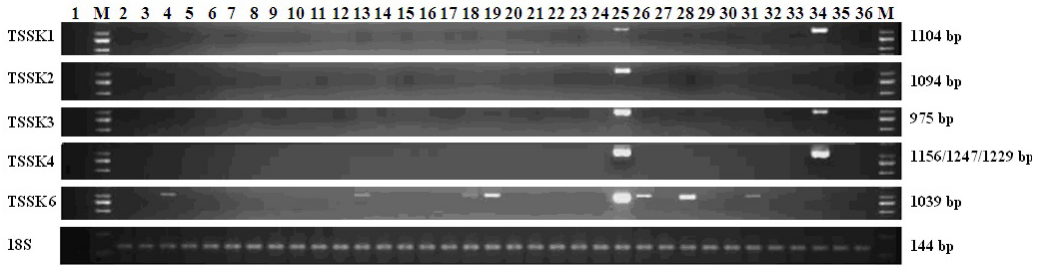

Figure 6. Expression patterns of porcine testis-specific serine kinase (*TSSK*) genes from 35 tissues of a 250-day-old pig. 18S rRNA was used as a control. *Lane 1* = blank; *lane 2* = cerebrum; *lane 3* = cerebellum; *lane 4* = hypothalamus; *lane 5* = brainstem; *lane 6* = spinal cord; *lane 7* = pituitary; *lane 8* = heart; *lane 9* = liver; *lane 10* = spleen; *lane 11* = lung; *lane 12* = kidney; *lane 13* = skin; *lane 14* = muscle; *lane 15* = duodenum; *lane 16* = jejunum; *lane 17* = ileum; *lane 18* = colon; *lane 19* = cecum; *lane 20* = rectum; *lane 21* = stomach; *lane 22* = pancreas; *lane 23* = esophagus; *lane 24* = lymph node; *lane 25* = testis; *lane 26* = epididymis; *lane 27* = submaxillary gland; *lane 28* = thyroid; *lane 29* = adrenal glands; *lane 30* = sublingual gland; *lane 31* = thymus; *lane 32* = conarium; *lane 33* = bulbourethral gland; *lane 34* = seminal vesicle; *lane 35* = prostate gland; *lane 36* = bladder; *lane M* = DL2000 DNA marker.

DISCUSSION

TSSKs are members of the AMPK superfamily and encode novel protein kinases with exclusive or predominant expression in testes. Specifically, male mice with *TSSK6* knock-out (KO) or double *TSSK1* and *2* KO are sterile, showing considerable reduction in sperm counts, impaired DNA condensation, abnormal morphology and impaired spermatozoa motility, which strongly suggests that TSSKs play an important role in normal germ cell differentiation and/or sperm function (Sosnik et al., 2009; Shang et al., 2010).

To date, although a large number of the *TSSK* genes have been found in various animals, many of them were identified through computational prediction methods based on sequence similarity. For example, among sheep and goat *TSSK* genes, only *TSSK6* has been experimentally tested, and other members were predicted computationally (Leng, 2012). For porcine *TSSK* genes, although *TSSK2*, *TSSK3*, *TSSK4*, and *TSSK6* have been predicted, all the members have not been experimentally confirmed. In this study, we cloned the full-length cDNAs of all porcine *TSSK* genes and further analyzed their gene structure and chromosomal location, then deduced the amino acid sequences based on their gene sequences and analyzed their evolutionary conservation. Furthermore, the expression profiles of these genes in different tissues were investigated using RT-PCR. To our knowledge, this is the first report to isolate and conduct spatial expression analysis of *TSSK* genes in swine.

From the results described here, the number of identified porcine *TSSK* genes is comparable with that of human and mouse, but there are some differences among these. For example, human *TSSK1* has one pseudogene, *TSSK1a*, which is not found in mouse and pig; human *TSSK4* only has two variants, but there are three variants in mouse and pig. Interestingly, mouse has a fifth member of the *TSSK* family, *TSSK5*, in the NCBI database, although it has not been experimentally confirmed. We also used this sequence to blast the pig nonredundant and high throughput genomic sequence database in the GenBank, but the homologous sequence was not found (data not shown). Therefore, the presence of a *TSSK5* member in the TSSK family requires further investigation. In addition, by comparison with the *TSSK4* gene in other species, we found that porcine *TSSK4* contains three alternatively spliced variants: *pTSSK4-1*, *pTSSK4-3*, and *pTSSK4-4*. *pTSSK4-1* is the same as the predicted sequence (XM_003128547) in NCBI at the nucleotide and protein level, while porcine *pTSSK4-2* (XM_003128548) predicted by computational approaches in NCBI was not detected in our study. This transcript may be expressed during a specific developmental stage or under special conditions, and we will further optimize our experimental procedures to determine whether this transcript is present in porcine. Porcine *pTSSK4-3* and *4* are new variants, which have not been ound previously.

Using amino acid alignment of TSSK4 proteins, we found that porcine TSSK4-3, human TSSK4-1, and mouse TSSK4-2 and 3 have an insertion of amino acid residues in region VIb of the HRD domain (His-Arg-Asp) compared with other TSSK4 proteins. The VIb region forms the catalytic loop in the phosphotransfer process and is important for normal kinase activity. *In vitro* kinase activity assays demonstrated that mouse TSSK4-2 and 3 showed no kinase activity, which suggests that these two variants are pseudokinases and the VIb region is crucial for kinase activity (Wei et al., 2007). Referring to results of mouse TSSK4, we can infer that porcine TSSK4-3 is also a pseudokinase and has lost its protein phosphorylation activity, but further experimental analysis is necessary to clarify this hypothesis.

From the tissue transcription pattern analysis in our study, it can be clearly seen that porcine *TSSK* genes are differentially expressed in adult tissues. *TSSK1*, *TSSK3*, and *TSSK4*

had a similar expression pattern, and were highly expressed in the testes and seminal vesicle compared with other tissue from the adult boar. *TSSK6* mRNA was expressed widely in a range of tissues: it was over-expressed in testes, moderately expressed in thyroid, cecum and epididymis, weakly expressed in lung, spleen and seminal vesicle, and almost silent in the other 24 tissues. Furthermore, *TSSK2* expression was confined to testes from the 35 tissues studied. The seminal vesicles can secrete a significant proportion of the fluid that ultimately becomes seminal fluid, which can dilute and provide nutrition to sperm. From the results presented here and their vital roles in sperm functions, we can infer that *TSSK* genes may be potential targets for male infertility screening and *TSSK2* is an ideal target for contraception. The relationships between *TSSKs* and male infertility traits in porcine should be further investigated.

Conflicts of interests

The authors declare no conflict of interest.

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