Alternative splicing, promoter methylation, and functional SNPs of sperm flagella 2 gene in testis and mature spermatozoa of Holstein bulls

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Abstract

The sperm flagella 2 (*SPEF2*) gene is essential for development of normal sperm tail and male fertility. In this study, we characterized first the splice variants, promoter and its methylation, and functional single-nucleotide polymorphisms (SNPs) of the *SPEF2* gene in newborn and adult Holstein bulls. Four splice variants were identified in the testes, epididymis, sperm, heart, spleen, lungs, kidneys, and liver tissues through RT-PCR, clone sequencing, and western blot analysis. Immunohistochemistry revealed that the *SPEF2* was specifically expressed in the primary spermatocytes, elongated spermatids, and round spermatids in the testes and epididymis. *SPEF2-SV1* was differentially expressed in the sperms of high-performance and low-performance adult bulls; *SPEF2-SV2* presents the highest expression in testis and epididymis; *SPEF2-SV3* was only detected in testis and epididymis. An SNP (c.2851G>T) in exon 20 of *SPEF2*, located within a putative exonic splice enhancer, potentially produced *SPEF2-SV3* and was involved in semen deformity rate and post-thaw cryopreserved sperm motility. The luciferase reporter and bisulfite sequencing analysis suggested that the methylation pattern of the core promoter did not significantly differ between the full-sib bulls that presented hypomethylation in the ejaculated semen and testis. This finding indicates that sperm quality is unrelated to *SPEF2* methylation pattern. Our data suggest that alternative splicing, rather than methylation, is involved in the regulation of *SPEF2* expression in the testes and sperm and is one of the determinants of sperm motility during bull spermatogenesis. The exonic SNP (c.2851G>T) produces aberrant splice variants, which can be used as a candidate marker for semen traits selection breeding of Holstein bulls.

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Introduction

The widespread use of dairy bull semen requires high sperm quality, which is economically important in the artificial insemination industry. During mammalian spermatogenesis, male germ cells undergo a series of differentiation steps that lead to the production of mature haploid spermatozoa. This complex physiologic process includes chromatin reorganization, cytoplasm elimination, acrosome formation, and flagellum development in the seminiferous tubules of the testis and epididymis (O'Donnell *et al.* 2001, Bettgowda & Wilkinson 2010). Normal sperm flagellum is essential for sperm motility and fertilization of the egg (Yanagimachi 1993). This process involves the coordinated expression of many genes with unique cellular and temporal specificities. Identifying the genes specifically expressed in newborn

and adult testes and sperm is crucial to understand testicular development and function, as well as spermatogenesis (Huang *et al.* 2004).

The sperm flagella 2 (*SPEF2*) gene, also known as *KPL2*, is essential for normal sperm tail development and male fertility. *Spef2* expression has previously been found to be stage specific and intensive in spermatocytes and round spermatids in the seminiferous tubules of rat testes (Ostrowski *et al.* 1999). *SPEF2* has been detected in both germ cells and Sertoli cells. The intense expression is located in the manchette, in the tail of elongating spermatids, and in the tail of the mouse sperm (Sironen *et al.* 2010). The loss of the *SPEF2* gene causes a decline in elongating spermatids during spermiogenesis and fault in the formation of sperm tail (Sironen *et al.* 2011). Previous studies have suggested that *SPEF2* has an

important role in the sperm tail formation and ciliary function involved in sperm motility. However, the molecular mechanisms of *SPEF2* gene expression regulation remain unknown. The developmental processes that act on male germ cells that culminate in the production of functional spermatozoa are regulated by alternative splicing (AS) and methylation mechanisms at the transcriptional, post-transcriptional, and epigenetic levels (Zamudio *et al.* 2008, Bettegowda & Wilkinson 2010, Schagdarsurengin *et al.* 2012). AS provides a versatile means of regulating gene expression using different combinations of exons from the same primary transcript, resulting in the generation of different mature transcripts and coding the same, shorter, or even distinct proteins (Elliott & Grellscheid 2006). About 5–45% of multi-exon genes undergo AS in different eukaryotes (Brett *et al.* 2000). AS is particularly prevalent in the testes and it plays an important role in several developmental pathways. Several studies have shown that deviations in aberrant transcripts are one of the causal factors in the reduced reproductive performance of bulls, including sperm maturation and fertilization (Erikson *et al.* 2007, Brandenburger *et al.* 2011, Noda *et al.* 2013). Single-nucleotide polymorphisms (SNPs) in exonic splice enhancers (ESEs) or exonic splice silencers are associated with spliced pre-mRNA by causing aberrant splicing in or near *cis*-acting elements, including exon skipping and/or intron retention; the mutation increases the frequency of the skipping of exons in the transcription process and gives rise to aberrant transcripts (Liegel *et al.* 2011). The bovine *SPEF2* gene is composed of 36 exons and 35 introns and is considered as a multi-exon gene. A retrotransposon inserts an *SPEF2* intron that causes aberrant splicing, which leads to immotile short-tail sperm in Finnish Yorkshire boar (Sironen *et al.* 2006). Therefore, we hypothesize that the expression of bovine *SPEF2* gene may be regulated by the AS mechanism and mutations of bovine *SPEF2* can lead to aberrant splice variants, which has potential roles in testicular development and spermatogenesis in bulls.

In mammals, DNA methylation is one of the most stable epigenetic modifications and is an important regulator in a number of biological processes, including testicular development and spermatogenesis (Oakes *et al.* 2007). Genetically, DNA methylation usually occurs in sequences with a CpG island, which are located on the promoter regions of genes in differentially methylated regions or in imprinting control regions (Bird 2002, Jaenisch & Bird 2003). Correct DNA methylation, however, has an important role in sperm production because hypermethylation has been associated with poor sperm parameters, idiopathic male infertility, and even pregnancy failure (Schagdarsurengin *et al.* 2012). Several genes, namely *MTHFR*, *PAX8*, *IGF2*, *KCNQ1OT1* (*LIT1*), and *SNRPN*, in the testes are regulated through epigenetic mechanisms and are

involved in poor semen parameters or male infertility (Rajender *et al.* 2011). During the selection process of excellent bulls with elite genetic potential and high semen performance, some of the full-sib bulls present diverse semen phenotypes. We deduced that the difference might be caused by epigenetic regulation. Therefore, we analyzed the promoter methylation pattern of the candidate *SPEF2* gene 5'-flanking region in the sperm cells from high-performance and low-performance adult bulls.

To confirm our hypotheses, the splice variants and their expression, as well as the localization of the *SPEF2* gene in Holstein bull tissues, including the testes, epididymis, and sperm, were investigated; the functional SNPs that caused splice variants and associated with semen quality traits were also detected; the promoter and methylation pattern of the *SPEF2* gene in ejaculated sperm and testis were also identified in full-sib paired Holstein bulls.

Subjects and methods

Ethics statement

All experiments were carried out according to the Regulations for the Administration of Affairs Concerning Experimental Animals published by the Ministry of Science and Technology, China in 2004 and approved by the Animal Care and Use Committee in Shandong Academy of Agricultural Sciences, Shandong, People's Republic of China. We obtained permission from the slaughterhouse and Shandong OX Biotech Co., Ltd. to use animal parts.

Semen and tissue samples

Experiment 1

Tissue samples from three newborn (2 days old) and three adult Holstein (3 years old), including testes, liver, heart, spleen, lungs, and epididymis, obtained from the Shandong commercial slaughterhouse were used to search the splice variants of the *SPEF2* gene using RT-PCR and clone sequencing methods.

Experiment 2

To further investigate the expression of different transcripts of bovine *SPEF2* gene in semen, fresh semen samples were obtained from 20 adult Holstein bulls (3–5 years old) in Shandong OX Biotechnology Co., Ltd. One ejaculate from each bull was collected using an artificial vagina, which was evaluated in terms of volume per ejaculate, sperm motility, sperm concentration, and percentage of abnormal sperm as described by Pan *et al.* (2013). Twenty bulls were assigned into two groups based on semen quality and age: a high-performance group and a low-performance group. According to the Frozen Bovine Semen Standard (GB/T 4143-2008, China), the semen that met the following criteria were included into the high-performance group (sperm motility $69.7 \pm 0.99\%$: sperm motility $\geq 65\%$, sperm concentration $\geq 6 \times 10^8/\text{ml}$, and

abnormal sperm percentage $\leq 15\%$. The remaining bulls were included into the low-performance group (sperm motility $51.3 \pm 1.86\%$). The fresh semen samples were maintained at 37°C and immediately returned to the laboratory for total RNA and DNA extraction. The tissue samples were immediately frozen in liquid nitrogen and used for total RNA and protein isolation. Fresh testicular and epididymal samples from two adult bulls were fixed in 4% paraformaldehyde for 48 h at room temperature, embedded in paraffin, and then cut into $7\ \mu\text{m}$ sections for localization of bovine SPEF2 protein.

Experiment 3

To investigate the promoter methylation pattern of the *SPEF2* gene, the core region of the *SPEF2* promoter was identified and fresh semen DNA samples from four full-sib paired bulls (Table 1) as well as another four adult bulls' testicular tissues were treated with sodium bisulfite. The samples were treated the same as in Experiment 2.

Experiment 4

A total of 109 Chinese Holstein bulls from three bull stations (Shandong OX Biotechnology Co., Ltd., (Jinan, Shandong, China) Beijing Dairy Cattle Center (Beijing, China), and Shanghai Bright Holstein Co., Ltd., Shanghai, China) were included in this study for the association analysis between genotypes and semen quality traits. The fresh semen quality traits, including semen volume per ejaculate (ml), and sperm concentration ($\times 10^8/\text{ml}$) and mobility were measured as mentioned in Experiment 2. After investigating the above traits, the fresh semen was diluted with glycerol–egg yolk–citrate mixture, packaged in 0.25 ml straws and cryopreserved. Two straws were randomly obtained from each sample, ejaculated and thawed at 38°C for 20 s after storage in liquid nitrogen for 5–7 days, and immediately evaluated for the frozen/thawed sperm motility under light microscopy, according to the criteria entitled Frozen Bovine Semen standard (GB/T 4143-2008, China).

Sperm DNA isolation

Genomic DNA was salted out from the semen. Briefly, 80 ml of lysis solution (12.5 mM EDTA, pH 8.0; 12.5 mM Tris–HCl, pH 8.0; 0.4 M dithiothreitol; 0.4 M NaCl; and 12.5% SDS) with 20% SDS and proteinase K (20 mg/ml) were added to the sperm pellet. The mixed liquor proceeded at 55°C for about 10 h. Then, the genomic DNA was separated using saturated NaCl solution and precipitated using precooled 100% ethanol.

The DNA pellet was washed twice in 500 μl of 75% ethanol and air-dried for a few minutes at room temperature. Finally, the DNA was dissolved in 100 μl of TB buffer (0.1 M Tris–HCl and 1 mM EDTA). The DNA was then stored at -20°C until subsequent analysis.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from semen following the same protocol. Briefly, the somatic cells in semen were lysed in 1 ml of cell lysis solution (0.1% SDS and 0.5% Triton X-100) and then washed in 1 ml of rinsing solution (60% Tris–OH and 8.6% sucrose). The sperm cells were lysed in 1 ml of TRIzol (Invitrogen) and proteins were removed using 200 μl of chloroform. Total RNA was precipitated with an equal volume of isopropanol, and the RNA pellet was washed with 75% ethanol. Finally, the total RNA was dissolved in 20 μl of DEPC water. cDNA was synthesized using a transcript first-strand cDNA synthesis kit (TaKaRa, Dalian, China). The total RNA extraction from tissues and RT into cDNA were performed according to a previously published protocol (Hou *et al.* 2012).

Identification of SPEF2 splice variants

To search potential splice variants of the bovine *SPEF2* gene, five primer pairs named S1, S2, S3, S4, and S5 (Table 2), which span the 5'-UTR and the 3'-UTR, were designed to amplify the full-length transcript and splice variants according to the bovine *SPEF2* reference sequence (GenBank: XM_616682). On the basis of the identification of splice variants, the primer pair SPEF2-1 (Table 2) was further designed to amplify the region spanning exons 29–33 for distinguishing the novel *SPEF2*-SV1 and complete *SPEF2* transcripts by agarose gel electrophoresis, and another primer pair SV3 (Table 2) was used to differentiate the *SPEF2*-SV2, *SPEF2*-SV3, and complete *SPEF2* transcripts. PCR was carried out using 12.5 μl of $2\times$ TaqPCR MasterMix (TransGen Biotech, Beijing, China), 0.5 μl of each forward and reverse primers (10 μM), and 1 μl of cDNA (100 ng/ μl), with the addition of ddH_2O to reach a total volume of 25 μl . PCR was carried out with an initial denaturing step at 94°C for 5 min and then 35 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 8 min. The PCR products were recovered from 1.5% agarose gel and purified using a GenClean Kit (Cwbiochem, Beijing, China) according to the manufacturer's protocol. Then, the purified products were cloned into the pGEMT-Easy vector (Promega) and transformed into *Escherichia coli* DH5 α cells. Individual clones were sequenced using an ABI 3730xl sequencing platform.

Table 1 Semen quality parameters and promoter methylation levels of the four full-sib bulls in Experiment 3.

Bull no.	Age	Volume of ejaculate (ml)	Sperm motility (%)	Sperm concentration ($10^8/\text{ml}$)	Percentage of abnormal sperm (%)	Percentage of promoter methylation (%)
1	4.5	7.08	55	14.67	19.42	8.02
2	4.5	8.96	73	17.06	9.05	5.56
3	6	6.47	53	10.29	20.33	2.78
4	6	9.09	72	12.41	8.26	5.56

Bulls 1 and 3 belong to the low-performance group; bulls 2 and 4 belong to the high-performance group; bulls 1 and 2 are full-sib, whereas bulls 3 and 4 are full-sib.

Table 2 Primer information of the bovine *SPEF2* gene.

Primer names	Sequences (5'–3')	Annealing temperature (°C)	Length (bp)
S1	Forward: AGGCAGGCGGGCTAGGTTTC Reverse: CCTTATCTCTGAGGGCTTGT	60.0	1117
S2	Forward: GAAGAACAAGCCCTCAGAGA Reverse: GAAGAACAAGCCCTCAGAGA	58.0	1121
S3	Forward: CCTCATAGAAGTGGAGGGAA Reverse: GCCTGCATCAGTGAGAAGAA	58.0	1229
S4	Forward: GGAGCGTCTGGACATCATT Reverse: CATTCAAGTACCTCTCACCG	57.0	838
S5	Forward: GATAGCTACACAGTCCGAC Reverse: GACCTCTCTTCATCACTTC	60.0	1352
SV3	Forward: AGATAAACAGTCATTGTGCC Reverse: CGGTGAAACATCTGTAGACT	55.0	396
G20	Forward: TAATCCTCCACCAGAATCTG Reverse: GCCTTATGATGTGGGTATGA	59.0	584
SPEF2-1	Forward: AGAAAACCAGCCAGCAGAAA Reverse: ACGTGAGTCCCAATGGCTAC	50.0	635
Q-SPEF2	Forward: GCTGGATTTGGTAACCCTGAA Reverse: GGTCTCCAGAAGCTCCTCCTC	63.0	189
Q-SPEF2-As	Forward: AGATCCACCTCCTCCGGTACG Reverse: AACCACAGGCCAGCTTCAG	59.0	233
β-actin	Forward: GCACAATGAAGATCAAGATCATC Reverse: CTAAAGTCCGCCTAGAAGCA	55.0	173
M-SPEF2-outer	Forward: AAAATTGAGGTTATTGGAGTAGGGG Reverse: AAAATACAAAACCTCAACCCTAA	55.3	285
M-SPEF2-inner	Forward: AGGTTATTGGAGTAGGGGTTTTATAA Reverse: CAAAACCTCAACCCTAAAAACAAA	55.3	271
pGL3-2004	Forward: CCGGGTACC GATTACAGCCAAGGAGTGC (KpnI) Reverse: CCGCTCGAGCAGGGGGCACAACTAATAG (XhoI)	61.9	2004
pGL3-1515	Forward: CCGGGTACC GGTCTGGGACTAAAGTTGT (KpnI) Reverse: CCGCTCGAGCAGGGGGCACAACTAATAG (XhoI)	60.1	1515
pGL3-1039	Forward: CCGGGTACCCTAGGAGGACTTTTGTGGC (KpnI) Reverse: CCGCTCGAGATTCTAACTGTCCAGGGGC (XhoI)	61.6	1039
pGL3-450	Forward: CCGGGTACCAGGCTAACCATGTCGGAGA (KpnI) Reverse: CCGCTCGAGCAGGGGGCACAACTAATAG (XhoI)	65.0	450
pGL3-829	Forward: CCGGGTACC CAAGAGTCTTCTCCAACACCA (KpnI) Reverse: CCGCTCGAGATCAGCTCGCTCGTTCCA (XhoI)	63.2	829
pGL3-463	Forward: CCGGGTACC TCCCTGCCTCCTCCTCCA (KpnI) Reverse: CCGCTCGAGATCAGCTCGCTCGTTCCA (XhoI)	68.5	463
pGL3-287	Forward: CCGGGTACC GCACCCTCTTCTCCAGCCT (KpnI) Reverse: CCGCTCGAGATCAGCTCGCTCGTTCCA (XhoI)	66.2	287

Multiple sequence alignment was performed to identify the splice variants using DNAMAN v5.2.2 and Chro-maspro1.41 (Technelysium, Helensvale, QLD, Australia) Software. We performed the following step to distinguish the detected splice variants from PCR and sequencing errors. First, positive clones were selected randomly from each sample and were resequenced using a commercial server (Invitrogen). Second, we validated the splice variant through RT-PCR and verified the new sequences by resequencing the RT-PCR cloning products.

Quantitative RT-PCR

Quantitative RT-PCR (RT-qPCR) was carried out to determine the mRNA relative expression of the *SPEF2* splice variants in the high-performance and low-performance bull semen. To determine the differential expression between *SPEF2*-complete and *SPEF2-SV1*, we designed the specific PCR primers Q-SPEF2 and Q-SPEF2-AS (Table 2 and Fig. 1a and b) and used cDNA produced from sperm-derived templates to amplify *SPEF2*-complete and *SPEF2-SV1* transcripts using the SYBR Green PCR Master Mix (Tiangen, Beijing, China) according to

the manufacturer's protocol. The primers for the housekeeping internal control β-actin gene that amplifies a 173 bp fragment were referred from a previous report (Hou *et al.* 2012). The 20 µl RT-qPCR mixture contained 10.0 µl of SYBR Premix 2× ExTaq II, 0.4 µM of the forward and reverse primers, 2.0 µl of cDNA (~100 ng) or plasmid DNA, and 6.4 µl of ddH₂O. The RT-qPCR was performed under the following conditions: 94 °C for 5 min, 40 cycles at 94 °C for 15 s, 56 °C for 15 s, and 70 °C for 5 s. The last stage for the dissociation curve was as follows: 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. The relative expression of the transcripts was calculated based on a previous report (Wang *et al.* 2012). Each sample was measured in triplicate and the experiment was repeated at least three times.

Prediction of promoter bioinformatics

The CpG islands were analyzed using the online software CpG Island Searcher (<http://www.uscnorris.com/cpgislands2/cpg.html>). We designed two bisulfite-modified specific PCR primer pairs, M-SPEF2-outer and M-SPEF2-inter (Table 2), for

Figure 1 RT-PCR of *SPEF2* splice variants expressed in different bull tissues. (a) Expression pattern of *SPEF2* transcripts was detected in newborn bulls. β -actin (173 bp) was used as the positive control. (b) *SPEF2* and *SPEF2-SV1* transcripts were detected in different tissues and ejaculate, which indicated that the *SPEF2* band (635 bp) is highly expressed in the testes and ejaculate of adult bulls. (c) Expression of *SPEF2* (396 bp), *SPEF2-SV2* (222 bp), and *SPEF2-SV3* (147 bp) transcripts in different tissues of adult bulls. The 396 bp band corresponds to the total *SPEF2* transcripts; Lane 1, heart; lane 2, liver; lane 3, spleen; lane 4, kidney; lane 5, testis; lane 6, epididymis; lanes 7–9, sperm of high-performance bulls; and lanes 10–12, sperm of low-performance bulls.

nested PCR amplification using the MethPrimer program (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>). The promoter of the bovine *SPEF2* gene was predicted using the Genomatix Software (<http://www.genomatix.de/applications/index.html>). The presence of a TATA box was predicted using PROSCAN, version 1.7 (<http://www.bimas.cit.nih.gov/cgi-bin/molbio/proscan>). Transcription factors were predicted using Promoter Scan (<http://www.bimas.cit.nih.gov/molbio/proscan/>) and TFSEARCH (ver.1.3; <http://www.cbrc.jp/research/db/TFSEARCH.html>).

Sodium bisulfite treatment

Genomic DNA was treated using a BisulFlash DNA modification kit (Epigentek, Brooklyn, NY, USA) according to the protocol of the manufacturer. Modified DNA was maintained at -20°C until PCR amplification.

PCR amplification, cloning, and bisulfite sequencing

PCR was carried out in a final volume of 20 μl . The nested PCR conditions were as follows: the reaction system for the first-round amplification consisted of 10 of $2\times$ GC buffer I (5 mM Mg^{2+} Plus), 3.4 μl of 2.5 mM dNTP mixture, 0.2 μl of TaKaRa LATAq (5 U/ μl), 1 μl of each 10 μM M-*SPEF2*-out primers, 3 μl of template DNA for the first round, 0.5 μl of template DNA (from the first round product), and M-*SPEF2*-inter primers for the second round amplification. The PCR program was as follows: initial denaturation at 94°C for 3 min; 45 cycles at 94°C for 40 s, 45°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 15 s. The PCR products were

detected on 1.5% agarose gel, purified using a Gel/PCR extraction kit (Biomiga, Beijing, China), cloned into the pGEM-T Easy Vector (TransGen Biotech), and then transformed into *E. coli* DH5 α cells for clone sequencing. Only the sequences derived from clones with $>95\%$ cytosine conversion were analyzed. The percentage of DNA methylation was calculated by counting the number of methylated CpGs out of the total number of CpG sites in individual clones. The bovine *SPEF2* sequence (GenBank: AC_000177.1) was used as a reference for methylation status analysis using the BiQ Analyzer Software.

Construction of *SPEF2* promoter-reporter plasmids

SPEF2 promoter activity was evaluated by dividing the region from g. -2064 to g. $+440$ into seven fragments, designated as pGL3-2004, pGL3-1515, pGL3-1039, pGL3-450, pGL3-829, pGL3-463, and pGL3-287. The primers contained the restriction enzyme site for KpnI and XhoI (Table 2). Each fragment was amplified, recovered, purified, and cloned into the pGL3-basic luciferase reporter vector according to a previously described protocol (Li *et al.* 2013). The sequences of these vectors were confirmed through direct sequencing on an ABI 3730xl sequencing platform.

Transient transfection and luciferase reporter assay

The murine Leydig tumor (MLTC-1) cell line was cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) containing 10 mg/l of penicillin and streptomycin (Invitrogen) at 37°C in a controlled humidified atmosphere with 5% CO_2 . The day before transfection, MLTC-1 cells were distributed in a 48-well plate. Upon reaching 75–85% confluence, the cells were cotransfected with 400 ng of the constructs or plasmid pGL3-basic and 50 ng of *PRL-TK* using Lipofectamine 2000 (Invitrogen). The plasmid pGL3-basic was used as the negative control and *PRL-TK* was used as the internal control to conform the difference in transfection. After 24 h of cultivation, the cells were washed and lysed in 1.5 ml tubes. A Dual-Luciferase Reporter Assay System (Promega) was used to analyze the luciferase activity according to the manufacturer's instructions. All transfections were performed in triplicate and repeated at least thrice in independent experiments. Promoter activity was analyzed relative to firefly luciferase activity normalized against Renilla luciferase activity.

Western blot analysis

The tissue samples from the newborn and adult bulls were homogenized using RIPA lysis buffer (Beyotime, Nantong, Jiangsu, China). After cooling the lysate on ice for 30 min, it was centrifuged at 10 000 g for 10 min at 4°C . The proteins were separated after denaturation via 12% SDS-PAGE, wet transferred onto a PVDF membrane, blocked with blocking buffer (Beyotime), and rotated for 1 h at RT. The blots were incubated with monoclonal anti-*SPEF2* (1:500; Abcam, Hong Kong, China) or polyclonal β -actin (1:500; Beyotime) for 2 h at RT. The *SPEF2* peptide sequences were based on the following murine part of *SPEF2*

(AQEEAYREEQLINRLMRQSQQERRIAVQLMHVRHEKEVLWQ-NRIFREKQHEERRLKDFQDALDREAALAKQAKIDFEEQFLK-EKRFHDQIAVERAARY), which has 90.9% homology with the corresponding bovine SPEF2 protein. After washing the membranes three times with 0.1% Tween-20 in $1 \times$ TBS for 5 min each time, anti-mouse or anti-goat secondary antibodies (1:500; Beyotime) were incubated with the membranes to detect antigen-antibody complexes. The bound secondary antibodies were visualized using a 3,3'-diaminobenzidine tetrachloride (DAB) HRP color development kit (Beyotime) according to the manufacturer's instructions. The molecular mass of the proteins was measured using a prestained protein ladder (Thermo, Waltham, MA, USA).

Immunohistochemistry

Testicular and epididymal tissues from two adult bulls were fixed in 4% paraformaldehyde. All tissues were then embedded in paraffin and sectioned for immunohistochemistry (IHC). A total of 1 l of deionized water and 10 ml of citrate buffer solution were used to rehabilitate the antigen and washed with 0.01 M PBS (pH 7.4) twice every 3 min. Then, the immunoreaction slides were deparaffinized and hydrated. The slides were blocked with endogenous peroxidase for 10 min and were subsequently washed with PBS and incubated with monoclonal anti-SPEF2 antibodies (1:50; Abcam) for 60 min at RT. After washing with PBS, the slides were incubated with anti-mouse secondary antibodies for 10 min at RT. The antibodies were visualized with 0.6 mg/ml DAB (Cwbiochem) HRP color development kit (Cwbiochem) for brown staining under a microscope (Leica LB30T, Wetzlar, Germany) according to the manufacturer's instructions. The slides were then stained with hematoxylin (Cwbiochem), dried again, and photographed using a digital camera (Leica).

SNP screening and genotyping

The primer pairs spanning from the exons 17, 18, 19, and 20 of the SPEF2 gene were used to amplify the fragment including the deletion of splice variants and to screen potential SNPs. The PCR amplification fragments were sequenced for SNP screening using an ABI 3730xl sequencing platform in the commercial server (Invitrogen). Only the primer pairs named G20 (Table 2) screened one SNP. Genotyping of the SNP was performed using the PCR-restriction fragment length polymorphism. The restriction endonucleases TaqI was selected to digest PCR products and the digested fragments were separated on 2% agarose gel.

Statistical analysis

Data are presented as mean or mean $\pm$ S.E.M. Statistical significance for gene expression and promoter activity was tested using a Student's *t*-test. The methylation patterns of the CpG sites of the SPEF2 promoter were analyzed using a χ^2 test.

The association between SNP genotypes and sperm quality traits was analyzed using the general least-square model procedure from SAS 9.0 (Statistical Analysis Software, SAS Institute, Cary, NC, USA). The line model is $Y_{ijkl} = \mu + G_i + A_k + P_j + H_l + e_{ijkl}$, where Y_{ijkl} is the observed value of each

semen quality trait; μ is the overall mean; G_i is the fixed effect of genotype; A_k is the fixed effect of age; P_j is the fixed effect of the origin of bull; H_l is the effect of farm; and e_{ijkl} is the random residual error. Differences between groups were considered significant at $P < 0.05$.

Results

Identification and expression of bovine SPEF2 splice variants

To analyze the possible splice variants of the SPEF2 gene among the different tissues from two developmental stages of bulls, primer S1, S2, S3, S4, and S5 was used to amplify the full-length coding region using cDNA as the template. We obtained the full-length transcript and the three novel transcripts through clone sequencing. To distinguish various transcripts using agarose gel electrophoresis, primers SPEF2-1, and SV3 spanning the deletion region were designed to amplify the shorter SPEF2 gene transcripts. The RT-PCR detected four amplicons in different tissues that were dependent on developmental stage (Fig. 1). SPEF2 mRNA was expressed predominantly in the testes of adult bulls and weakly expressed in other tissues. Relatively higher signals were also detected in the epididymis and the ejaculate. Intriguingly, only one 635 bp band was detected in the lungs, whereas two bands were detected in other tissues in newborn bulls (Fig. 1a). In adult bulls, we also detected SPEF2-complete and SPEF2-SV1 in the kidneys, lungs, spleen, liver, heart, testes, and semen (Fig. 1b). The two main bands, 635 and 452 bp, were purified and cloned into the pGEM-T Easy vector for sequencing. As shown in Fig. 2, the 635 bp fragment corresponds to the total transcripts, whereas the 452 bp fragment belongs to a novel SPEF2-SV1 splice variant that lack a 183 bp of exon 31. The 396, 222, and 147 bp fragments corresponded to the total SPEF2, novel SPEF2-SV2, and SPEF2-SV3 transcripts respectively (Fig. 1c). The expression level of SPEF2-SV2 transcript in testis was more abundant than in other tissues. Interestingly, the SPEF2-SV3 was only detected in testis and epididymis (Fig. 1c). SPEF2-SV2 and SPEF2-SV3 delete exon 18 and exon 18+exon 19 respectively (Fig. 2). Three novel transcripts were predicted to encode the truncated proteins with 1710, 1713, and 1688 aa, without an introduction of open reading frame shifting. As shown in Fig. 3, the complete bovine SPEF2 encodes a six-domain protein that includes two calponin homology domains, one actin-binding formin homology 2 domain (FH2), an adenylate kinase site, and UDF domains, whereas, the novel transcript SPEF2-SV1 (deposited in GenBank: KF425520) encodes a truncated protein with a deleted EF-hand domain, which affects the SPEF2 activity through calcium regulation; SPEF2-SV2 (deposited in GenBank: KF733181) and SPEF2-SV3 (deposited in GenBank: KF733182) missed the FH2.

Figure 2 Diagram depicting the genomic organization and splice variants of the bovine *SPEF2* gene. The primers *SPEF2*-F and *SPEF2*-R were used to identify new transcripts; Q-*SPEF2*-F, Q-*SPEF2*-R, Q-*SPEF2*-As-F, and Q-*SPEF2*-As-R were used for RT-qPCR; Five primer pairs S1 (1F, 1R), S2 (2F, 2R), S3 (3F, 3R), S4 (4F, 4R), and S5 (5F, 5R) were used for amplification of the *SPEF2* coding region; SV3-F and SV3-R were used for distinguishing *SPEF2*-SV2 and *SPEF2*-SV3. Solid arrows indicate the position of primers; dashed-line arrows represent the location of SNPs; and numbers under the line indicate the position of *SPEF2* splicing deletions.

Expression and localization of bovine *SPEF2* protein

The bull tissues were subjected to western blot analysis to validate the expression and localization of *SPEF2*. The results show that *SPEF2* proteins ~200, 190, 180, 100, 50, and 37 kDa were expressed in the newborn and adult bull tissues, including the testes, epididymis, spleen, kidneys, liver, heart, and lungs (Fig. 4). Five *SPEF2* protein bands with different molecular weights were detected in the western blot analysis using antibodies against the 287 bp fragment from exons 6 to 8 of the bovine *SPEF2* gene. Thus, a ~200 kDa band was detected in the testicular samples and at lower levels in the epididymis (Fig. 4). Another four low-molecular-weight bands were also visible in the detected tissues, which may correspond to other possible bovine *SPEF2* isoforms, degradation products of the long *SPEF2* isoforms, or even cross-reactive products. Four bands were detected in the testes compared with other tissues. The 190 kDa band was predominantly expressed in the testes. Interestingly, 180 and 50 kDa tissue-specific bands were also detected in the heart. We detected a 100 kDa band in the liver of adult bulls. In newborn bulls, the full-length *SPEF2* protein (200 kDa) was detected only in the testes and no band was found in the spleen (Fig. 4a). The expression pattern of *SPEF2* in the heart was similar to that in adult bulls, and the 180 kDa band was absent in the liver and kidneys (Fig. 4b). This result shows that the *SPEF2* protein is differentially expressed in the testes at two

developmental stages and it may have a potential role in spermatogenesis in Holstein bulls. The two proteins, ~200 and 190 kDa, are consistent with the predicted molecular weights of the *SPEF2*-complete (202 kDa) and *SPEF2*-AS protein (~195 kDa). Although we were unable to distinguish the other proteins in the western blot analysis, we identified an AS variant of *SPEF2* that is transcribed in the spermatogenic cells of the testes and epididymis of bulls.

Figure 3 Sketch map of the amino acid sequences and domains of the bovine *SPEF2* protein. Compared with complete protein (1771 aa), *SPEF2*-SV1, *SPEF2*-SV2, and *SPEF2*-SV3 isoforms lacked the EF-hand, FH2, and FH2 domains (SWISS-model) respectively. The numbers indicate the position of the amino acids.

Figure 4 Expression and localization of the bovine SPEF2 protein. (a) Western blot analysis of SPEF2 in adult bull tissues using β -actin as the control. (b) Western blot analysis of SPEF2 in newborn bull tissues. (c) Immunolocalization of SPEF2 in adult bull's seminiferous epithelium and epididymis. (A, C, E, and G) Localization of SPEF2 in testis, caput epididymis, corpus epididymis, and cauda epididymis of adult bull; (B, D, F, and H) correspond to the negative controls. Brown indicates the expressed protein, whereas the blue is negative. Images were obtained at a 50 μ m scale plate.

To further understand SPEF2 protein expression during spermatogenesis, the testicular and epididymal morphologies of the bulls were analyzed via IHC. SPEF2 immunoreactivity was detected in the Sertoli cell and the seminiferous epithelium, including pachytene spermatocytes, primary spermatocytes, and spermatids (Fig. 4c). The immunoreactive pattern of the elongated spermatids was similar to that found in mice (Sironen *et al.* 2010). The IHC revealed SPEF2 protein expression in epithelial cells throughout the bull epididymis, especially in the corpus epididymis. The SPEF2 protein was located in the tall columnar epithelium with long microvilli in the corpus epididymis; however, a weak signal was detected in the caput epididymis and the cauda epididymis. We also observed abundant sperm in the lumen of adult cauda, which presented a feeble signal.

Relative mRNA expression patterns of SPEF2-SV2 in bull testes, epididymis, and semen

The RT-qPCR result shows that *SPEF2* splice variants were abundant in the bull testes, epididymis, and mature sperm. The *SPEF2*-complete mRNA expression in the testicles, epididymis, and semen of adult bulls was significantly higher than that of the *SPEF2*-SV1 ($P < 0.05$), which indicates that the long transcript is the main transcript in the three tissues. The total *SPEF2* and *SPEF2*-SV1 mRNA expression in the high-performance bull semen was significantly upregulated compared with that in the low-performance group, while *SPEF2*-SV3 presents the inverse expression tendency in semen. The *SPEF2* splice transcripts are therefore potentially involved in spermatogenesis.

Cloning and activity analysis of SPEF2 promoter

We found a promoter in the 5'-flanking region and the other six promoters in the bovine *SPEF2* gene, using bioinformatics software (data not shown). We first focused on the promoter in the 5'-flanking region and amplified seven fragments through progressive deletion of nucleotides from the 5'-end. Each fragment was cloned into the pGL3-basic luciferase reporter vector and then transiently transfected into MLTC-1 cells respectively. The relative luciferase activity of the promoter pGL3-1039 fragment was upregulated by ~ 10.3 -fold compared with that of pGL3-1515 and by ~ 5.4 -fold compared with that of pGL3-450 (Fig. 5a). The result indicated negative regulatory elements in the region from g.-1564 to g.-586. Several inhibitory transcription element-binding sites, such as *E2F* and *AML1a*, which inhibit the transcriptional activity of genes (Uchida *et al.* 1997, Ousephe *et al.* 2012), were found using the TFSEARCH Online Software (<http://www.cbrc.jp/research/db/TFSEARCH.html>) in the

Figure 5 Relative luciferase activity of the *SPEF2* 5'-flanking promoter in MLTC-1 cells. (a) Deletion fragment from g. -1564 to g. -10. The 5'-flanking region was divided into four fragments and cloned into PGL3-basic vector. The relative luciferase activity of each recombinant vector is indicated right of the fragment. (b) Deletion fragments from g. -699 to g. -157. To confirm the core region of the promoter, the PGL3-1039 fragment, which indicates relatively high activity, was divided into three fragments and cloned into PGL3-basic vector. The relative luciferase activity is also presented to the right of the fragment. PGL3-basic was used as a negative control.

region from g. -1066 to g. -586. To shorten the core promoter region, we divided the region from g. -699 to g. +130 into three fragments and cloned them into the pGL3-basic luciferase vector. These recombinant vectors were transiently transfected into MLTC-1 cells for luciferase activity analysis. Figure 5b shows that the promoter named pGL3-829 activity was significantly higher than that of pGL3-287 and pGL3-463 ($P < 0.05$). This result indicates that the region from g. -586 to g. -157 is the core promoter responsible for most of the promoter activity in the bovine *SPEF2* gene.

DNA methylation of the *SPEF2* promoter

To test whether the *SPEF2* promoter in the bull was transcribed normally, the DNA methylation patterns of *SPEF2* in the semen of four full-sib bulls were detected using bisulfite sequencing. We detected a CpG-rich domain in the core promoter using the online software CpG Island Searcher. Using the quantitative bisulfite sequencing method, we amplified the 271 bp fragment with nine CpG sites. In this study, 60 clones were evaluated, with each clone containing nine CpG sites within the amplified fragment of promoter analyzed. The percentage of the analyzed sequences in all clones were hypomethylated (<50% of CpG sites on a given methylated strand). No differences in the sperm DNA methylation of the *SPEF2* promoter were found between the high-performance (5.40%, $n = 30$ clones) and low-performance (5.56%, $n = 30$ clones) bulls (Table 1). However, the percentage of promoter methylation of bull 3 in the high-performance group was the lowest, which indicate individual differences among the four bulls.

Sperm nucleus is highly condensed, resulting in transcriptional repression. Thus, the transcription of the *SPEF2* gene is expected to mainly take place in spermatogenic cells in the testis. We also detected the promoter methylation status of *SPEF2* in the testis of four adult bulls. They also showed a hypomethylation level (mean value: 10.26%, $n = 50$ clones) in the testis tissues.

Relationships among functional SNPs, aberrant splice variants, and bull semen quality traits

Several studies showed that AS could be caused by the exonic SNPs located in the potential ESE motif (Cartegni *et al.* 2002). Consequently, we identified the mutations of the exons that are close to the region producing the aberrant splicing. As expected, we found a synonymous SNP (c.2851G>T) in exon 20 of *SPEF2* and a nonsynonymous SNP (c.3548T>C) in exon 25 in Chinese Holstein bulls. Using ESEfinder 3.0 Software (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi>), we predicted the change in ESE using the c.2851G>T mutation for it near the splicing region of *SPEF2*-SV3 and found that it increased one binding site for the splicing factor SRp40 (Fig. 6).

To verify whether the SNP (c.2851G>T) is associated with the production of the novel splice variants, we genotyped six bulls and also identified their corresponding *SPEF2*-SV2 and *SPEF2*-SV3 splice variants. As a result, the *SPEF2*-SV3 transcript was not found in the bulls with the GG genotype.

Figure 6 Potential ESE motif threshold scores of different genotypes of the *SPEF2* gene. Four different SR proteins (SF2/ASF (red), SF2/ASF (IgM-BRCA1) (pink), SC35 (blue), and SRp40 (green)) were predicted in sequence (GGTCCCAGTAGTGCCAGCCCCATCG/TAAGCCCGGAT). The underlined nucleotide indicates the SNP located in the binding sites for splicing factors.

Figure 7 Genotyping of the SNP (c.2851G>T) of the bovine *SPEF2* gene. PCR products were digested with TaqI; TT, TG, and GG genotypes present one band (584 bp), three bands (584, 460, and 124 bp) and two bands (460 and 124 bp) respectively. The digested *SPEF2* fragments were separated by 2% agarose gel electrophoresis.

To analyze the effect of the novel genetic variation of *SPEF2* on sperm quality traits (ejaculate volume, sperm density, and thawed sperm motility), the association analysis was performed in a population of 109 Chinese Holstein bulls. The result showed that the bulls with the GG genotype had lower deformity rates than those of the GT and TT genotypes, and that the GG genotype had significantly higher thawed sperm motility when compared with the bulls with the GT and TT genotypes. However, no significant differences in ejaculate volume and fresh sperm density were found (Table 3 and Fig. 7).

Discussion

SPEF2 splice variants are differentially expressed in the bovine testis, epididymis, and semen and are involved in sperm motility and function

We determined that the *SPEF2* gene has three novel transcripts that lack one or two exons in Holstein bulls. Of which, the novel transcript *SPEF2*-SV1, generated through AS, was expressed in the sperm cells, epididymis, testes, heart, spleen, and kidneys of newborn and adult bulls, and in the lungs of newborn bulls. Moreover, it was differentially expressed in the high-performance and low-performance bull semen. Further, the localization of *SPEF2* in the testes and epididymis suggests a role in spermatogenesis. These results indicate that *SPEF2* splice variants are involved in the development of testis and spermatogenesis in dairy bulls. They can play roles by gene AS mechanisms. Its abundant expression in epididymal epithelium is consistent with its role in bovine sperm maturation. Two sequence variants in the gene encode mouse *SPEF2* proteins,

namely flagellar assembly and cilia function, which are involved in spermatogenesis (Sironen *et al.* 2011). The *SPEF2* variants exhibited tissue-specific, phage-specific, and species-specific expression. In rats, *SPEF2* is expressed in tissues containing cilia-like structures, such as the lungs, trachea, testes, and the brain, at lower levels in the kidneys and spleen, and is not expressed in the heart and liver, which suggest its role in ciliogenesis (Sironen *et al.* 2010). In pigs, the *SPEF2* gene is differentially expressed in abnormal and healthy animals. Thus, the region coding for the C-terminal part of the protein appears to be expressed only in the testes and trachea, whereas the region coding for the N-terminal part is expressed in the lungs, liver, and kidneys (Sironen *et al.* 2006). *SPEF2* genes participate in spermatogenesis through AS in several species. In this study, we identified three novel splice variants via RT-PCR and clone sequencing; other potential splice variants need to be confirmed in the next experiment. The western blot analysis results imply that the bovine *SPEF2* gene is a multifunction gene that has broad functions even though the current study lacked specific antibodies for bovine isoforms. Previous research has reported that the presence of an insert retro-transposon within an intron causes immotile short-tailed sperm in Finnish Yorkshire pigs (Sironen *et al.* 2006). In rats, the testis-specific long *SPEF2* variant and transcripts containing exons 6–43 were detected (Sironen *et al.* 2010). Fifteen ASs of the human *SPEF2* gene were deposited in the Ensemble database (ENSG00000152582). They encode 12 different proteins through exon skipping and retained intron splicing patterns, and two splice variants were identified.

5' Flanking promoter of SPEF2 is hypomethylated in ejaculated semen and its methylation pattern is unrelated to sperm quality

DNA methylation defects in genes are reportedly associated with impaired human sperm production and quality (Navarro-Costa *et al.* 2010, Krausz *et al.* 2012). Whether these differences in semen quality between full-sib bulls reflect differences in DNA methylation pattern has not been addressed yet. We determined the methylation status of the *SPEF2* gene promoter. Methylation of the 5' promoters of DNA suppresses gene expression. In this study, the 5' promoter methylation pattern is consistent with the expression pattern of the *SPEF2* gene in the semen of the full-sib bulls, which is a

Table 3 Genetic effect of the SNP (c.2851G>T) on semen quality traits in Chinese Holstein bulls.

Genotype/sample	Allelic frequency	Ejaculate volume (ml)	Deformity rate (%)	Sperm density ($\times 10^8$ /ml)	Thawed sperm motility (%)
GG/16	G(0.42)	5.93 $\pm$ 0.45	15.60 $\pm$ 0.02 ^b	10.46 $\pm$ 1.78	43.32 $\pm$ 3.02 ^a
TG/59		5.78 $\pm$ 0.23	16.3 $\pm$ 0.02 ^b	12.15 $\pm$ 0.93	38.68 $\pm$ 1.59 ^{a,b}
TT/34	T(0.58)	5.62 $\pm$ 0.31	20.42 $\pm$ 0.02 ^a	13.11 $\pm$ 1.23	35.42 $\pm$ 2.10 ^b

Least-square mean indicated by different small letter superscripts within the same column differ significantly ($P < 0.05$).

good animal model for epigenetic analysis. During germ cell development, mammalian cells undergo nearly complete reprogramming of DNA methylation patterns. The majority of the promoters in sperm escape methylation, whereas the corresponding hypomethylated regions show substantial structural differences (Molaro *et al.* 2011). In mammals, tissue- and cell-specific methylation occurs in a small percentage of 5' CpG island promoters, whereas a far larger proportion occurs across gene bodies. Intragenic methylation is involved in the regulation of cell context-specific alternative promoters in gene bodies (Maunakea *et al.* 2010). In this study, the DNA methylation profile of the *SPEF2* promoter was unrelated to bull sperm quality. However, several promoters in the gene body were predicted in the bovine *SPEF2*; therefore, we cannot exclude the possible existence of other methylation patterns in bull sperm.

An exonic SNP (c.2851G>T) potentially produces aberrant splice variants and is associated with semen quality traits

Although the mechanisms of AS have been studied extensively, we have not understood fully the diversity and complexity of regulation. Increasing evidence shows that silent SNPs can affect mRNA splicing or mRNA levels/stability, leading to disease in humans (Cartegni *et al.* 2002). In our study, we identified three novel transcripts and an SNP that is located in the potential ESE motifs. The ESE can interact with specific serine/arginine-rich (SR) proteins, and they have diverse and critical functions in alternative pre-mRNA splicing. Using the ESEfinder 3.0 Software prediction, the T allele did not change the affinity with SF2/ASF, SF2/ASF (IgM-BRCA1), or SC35; however, it increased one potential binding site for the splicing factor SRp40. SRp40 being the splicing protein was reported to having a relationship with the unusual alternative splicing (Patel *et al.* 2005, Wang *et al.* 2012). We further found that the TT genotype of the SNP (c.2851G>T-G) could produce *SPEF2-SV3* aberrant transcript, while the GG genotype did not. Therefore, we speculate that the mutation may participate in the splicing process of the *SPEF2-SV3* splice variant. The overexpression of *SPEF2-SV3* transcript lacking the FH2 domain may partially affect sperm cilia formation and flagella assembly, which is needed for further investigation. Moreover, the results of association analyse also suggested that the SNP have significant effects on deformity rates and thawed sperm motility. We will construct plasmids containing different genotypes and transfect them into cells using the exon capture mini-gene system and further verify whether the SNP can cause *SPEF2-SV3* transcripts in the next study.

In summary, AS, rather than methylation, plays a role in the regulation of *SPEF2* expression in the testes and sperm. The exonic SNP (c.2851G>T) potentially leads

to aberrant splice variants and is associated with semen deformity rate and thawed sperm mobility. Analysis of the expression and regulation mechanism as well as SNP genetic effect of the *SPEF2* gene will improve our understanding of testicular development and spermatogenesis at the molecular level and may provide insights into candidate genes that determine the semen quality of Holstein bulls.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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