Japanese Journal of Veterinary Research 65(1): 5–15, 2017

JJVR

REGULAR PAPER Experimental Research

Identification of novel alternative splicing variants within swine *Setd8* gene and their high mRNA expression in testis

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Received for publication, August 26, 2016; accepted, November 28, 2016

Abstract

SET domain containing (lysine methyltransferase) 8 (Setd8), a histone modification enzyme, affects cell cycling, chromosome condensation, high efficient repair of DNA double strand breaks and so on. The objective of this study was to identify novel alternative splicing variants of pig *Setd8* gene and its mRNA expression. Four 180-day-old male Guanzhong Black (GZB) pigs and six male Landrace piglets (including three 30-day-old and three 7-day-old pigs) were collected to study *Setd8* gene. Herein, two novel variants, *Setd8a* and *Setd8b*, were found in pig. The entire sequences of *Setd8a* and *Setd8b* variants were 1,039 bp and 958 bp, respectively. qRT-PCR results showed that *Setd8a* and *Setd8b* were highly expressed in brains and testes of 180-day-old GZB pigs. Moreover, the expressions of the two *Setd8* variants were significantly higher in testis than brain of GZB pig ($P < 0.05$). Further study on testis showed that the mRNA expression of *Setd8a* variant was significantly lower than *Setd8b* variant in 30-day-old and 7-day-old pigs ($P < 0.05$). The mRNA expression of *Setd8a* variant was lower than *Setd8b* variant in GZB pigs ($P > 0.05$). Moreover, the expressions of the two *Setd8* variants were significantly higher along with age enlargement. In conclusion, *Setd8a* and *Setd8b* were firstly identified in pigs and both were expressed in pig testis. *Setd8b* was the major splicing variant of pig *Setd8* gene transcript product. Moreover, the expressions of *Setd8* variants were time-dependent. All these findings would enrich the study of *Setd8* gene in pig testis.

Key Words: pig, testis, SET domain containing (lysine methyltransferase) 8 (Setd8), alternative splicing variant, expression patterns.

Introduction

Pig is not only an important economic large domestic animal for meat industry, but also an ideal mammalian model for human biomedical and diseases studies[13,33,42)]. At present, pig industry in China faces severe problems, and one of the worst is subfertility. One way to solve male subfertility is spermatogonial stem cells (SSCs) transplantation[3,17)]. SSCs can transmit genomic information to the offspring by differentiating into spermatozoa[43)]. Thus it is worthy to study the biological characteristics and regulation mechanisms of SSCs. Present studies show

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doi: 10.14943/jjvr.65.1.5

that some critical factors, such as Wnt or proliferating cell nuclear antigen (PCNA), are relevant to regulating the proliferation and stem cell properties maintenance of SSCs[11,12,45,46]. Furthermore, the Wnt and PCNA expression are both mediated by SET domain containing (lysine methyltransferase) 8 (Setd8)[24,39,41]. Thus it could be conjectured that Setd8 might regulate the SSCs activity in mammals. However, little was known about function of *Setd8* gene in pigs.

Setd8, also known as *KMT5a*, *SET8* and *PR-Set7/9* gene, is located on pig chromosome 14. The *Setd8* gene in pig is 18,955 bp in length and contains eight exons and seven introns with strictly conserved intron/exon boundaries (NC_010456.4). As one member of SET domain protein superfamily, Setd8 has one SET domain, which consists of three elements: Suppressor of variegation 3-9 (Su(var) 3-9), Enhancer of zeste (E(z)), and Trithorax (Trx)[35]. The SET domain has a conserved sequence among the superfamily, and approximate 130 amino acids in length[36].

Setd8 has the irreplaceable function in human, mouse and other mammals as a histone modification enzyme[8,24]. Firstly, Setd8 was the exclusive enzyme catalysing histone H4 monomethylation on Lys 20 (H4K20me1). Secondly, present studies predicted that Setd8 could regulate cell cycling on S phase progression and promote chromosome condensation in cell cycle progression[15,25,37]. The absence of Setd8 was shown to be able to induce DNA damage, cell cycle arrest, and sometimes cell apoptosis[34,44]. It was suggested that the mouse embryo could not survival to birth when *Setd8* was deleted[32]. Moreover, latest study demonstrated that *Setd8* took a part in self-renewal of human adult stem cells[8]. Meanwhile, the expression level of DNA methyltransferase influenced the spermatogenesis[40]. Moreover, the expression of *Setd8* was decreased along with spermatogonia differentiating into spermatocyte in mouse[14]. Therefore, it could be hypothesized that Setd8 was one of the most important protein that support vital movement of pigs and taken effects on reproduction traits.

Histone modification was found to link with alternative splicing (AS) and AS played a part in vital activity of histone modification in turn[2,18]. *Setd8* had been found to have two certain AS variants and several predicted variants in human, and four predicted variants in mouse and rat[1,10]. However, variants of *Setd8* had not been reported in pig. To better understanding pig *Setd8* gene and its role in SSCs activity, we chose it as a candidate gene to explore whether AS would exist in pig testis or not and analyzed their mRNA expression. The data would contribute to understanding the role of *Setd8* gene in reproduction traits in pig, and further facilitate the development of pig industry.

Material and methods

Tissues collection: Use of all experiments animals and operation programs were permitted by International Animal Care and Use Committee of Northwest A&F University in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Four 180-day-old male Guanzhong Black (GZB) pigs and six male Landrace piglets (including three 30-day-old and three 7-day-old pigs) were collected from local farm and Besun agricultural industry group Co., Ltd. in Shaanxi province, China, respectively. A total of seven tissues (including heart, liver, spleen, lung, kidney, brain, and testis) were obtained from four GZB pigs. Only testicular tissues were collected from Landrace pigs. Each tissue was immediately submerged in liquid nitrogen and then stored at −80°C for subsequent study[22].

RNA extraction and cDNA synthesis: Total RNA of each sample was extracted using RNAiso Plus reagent (TaKaRa, Dalian, China), as well as the RNase-free DNase I (TaKaRa) was used to clean up genomic DNA from the RNA samples. The quantity of RNA was assessed by $OD_{260/280}$ value

Table 1. Primers used for cloning and expression analysis of pig *Setd8* and its variants

Primer	Primer sequences (5′–3′)	Reference sequence	Length of production/bp	Notes
P1	F: ATGACTAAACCTTCCGAG R: TGAGGGCACTTTGTCCAT	XM_013982772.1	1094/1013	mRNA sequence of *Setd8a*/*Setd8b*, respectively
P2	F: GTGTCCTTCCAGTGCAACCT R: AGAGCATTTGTTCGGGCTCA	KX254435	97	qRT-PCR for *Setd8a*
P3	F: GACTAAACCTTCCGAGGCGG R: ACTGAGTTCTCTTCCGTCGC	KR021363	124	qRT-PCR for *Setd8b*
GAPDH	F: ACACTCACTCTTCTACCTTTG R: CAAATTCATTGTCGTACCAG	NM_001206359.1	90	qRT-PCR for internal control

using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and agarose electrophoresis[22]. Then reverse transcription PCR was performed to synthesized cDNA using the PrimeSript™ RT reagent Kit (TaKaRa) according to the manufacturer's recommended procedure[23].

Identification of Setd8 splicing variants: On the basis of the predicted nucleotide sequence of pig *Setd8* gene (XM_013982772.1), a pair of primers (P1, Table 1) was designed by Primer Premier 5 software (Premier BioSoft, Palo Alto, CA, USA) to identify novel variants. PCR reactions were performed with Touch-Down PCR in a 25 μL volume containing cDNA, 0.5 μM of each primers (P2 and P3), 2×Eco Taq PCR SuperMix (+dye) *Taq* DNA polymerase, $MgCl_2$, dNTPs, and buffer and rest volumes of distilled water (Beijing TransGen Biotech Co., Ltd)[6]. The PCR products were analyzed by 2% agarose electrophoresis and the target bands were then purified with Gel Extraction Kit (Sangon Biotech, Shanghai, China). After that, the PCR products were sub-cloned into pMD19-T Vector (TaKaRa), and transferred into *Escherichia coli*. Competent cells *DH5α* (TaKaRa), then verified by sequencing (Gen-Script Co., Ltd, Nanjing, China).

Bioinformatics analysis: The sequences alignments of nucleotide were conducted by BioXM 2.6 (Nanjing Agricultural University, Nanjing, China) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast).

Measurement of Setd8a and Setd8b mRNA expression: According to the sequencing results, two pairs of primers (Table 1) were used to detect the mRNA expression levels of the two *Setd8* splicing variants. The house keeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Table 1) was used as internal control[20]. Quantitative real-time PCR (qRT-PCR) was run on a Bio-Rad IQ5 Real-Time PCR system with three repeats for each sample. In addition, a blank control was set in each sample group. The PCR reaction system was 20 μL in volume: 10 μL SYBR® Premix Ex Taq II (TaKaRa) (2×), 1 μL cDNA (diluted for 100 times), 0.5 μL of each primer (P2 or P3) (10 μmol/L) and rest volumes of distilled water.

Statistical analysis: The mRNA expressions of *Setd8* variants were computed by $2^{-\Delta\Delta CT}$ method, and normalized by the expression of *GAPDH*[32]. The mRNA expression variation among different samples was calculated by SPSS (version 18.0) (SPSS, Inc., Chicago, IL, USA). Statistical differences of *Setd8* variants expressions in different tissues and different ages were performed by ANOVA[44].

Results

Identification of pig Setd8 gene variants

Two variants were identified in pig, *Setd8a*

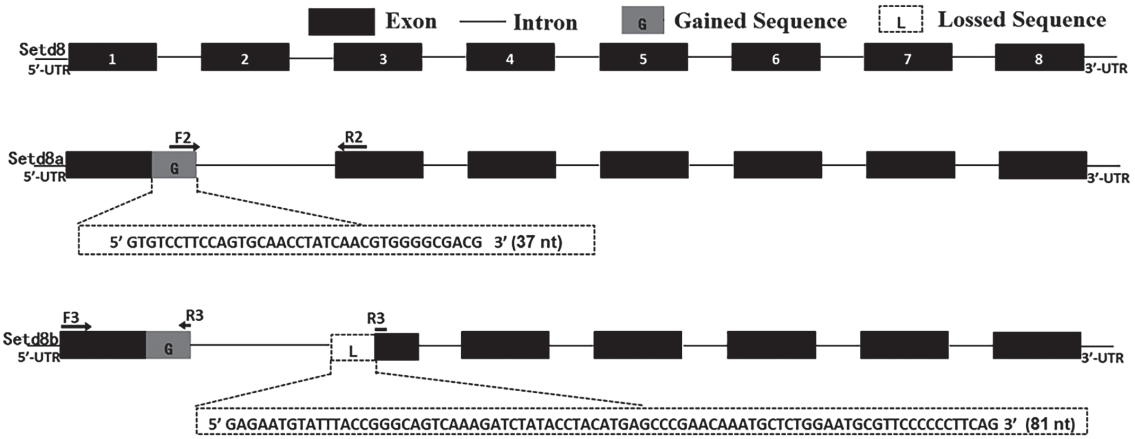

Fig. 1. The genomic structure diagram, positions of primers and the predictive transcription factors binding sites in swine *Setd8* and its variants. Note: The schematic used predicted *Sus scrofa Setd8* gene (XM_013982772.1) as reference sequence. *Setd8a* (KX254435) and *Setd8b* (KR021363) were identified in this study. The arrowhead represented the position of the primers of *Setd8a* and *Setd8b* variants (Primer 2 and 3).

(GenBank Accession number: KX254435) and *Setd8b* (GenBank Accession number: KR021363), and both were novel transcript variants found in swine in this study. The entire coding sequences of *Setd8a* and *Setd8b* were 1039 bp and 958 bp in length, respectively (Fig. 1). After confirming the two alternative splicing variants, these fragments were sub-cloned to pMD19-T Vector and verified by sequencing.

The nucleotide sequencing analysis found that *Setd8* had eight exons, while both *Setd8a* and *Setd8b* had seven exons, lacking of exon 2 (150 nucleotides). The exon 1 of *Setd8a* and *Setd8b* was 37 nucleotides (nt) longer than that of *Setd8*, and the 37 nt was from intron 1 retention (Fig. 1). When the 37 nt sequence was mapped to pig genome sequence, the splicing sites were found to comply with the GT-AG rule for 5' splice donor and 3' splice acceptor sites. The exon 3 of *Setd8b* was 81 nt shorter than those of *Setd8* and *Setd8a*, as well as the exon skipping was fit the GT-AG rule (Fig. 1).

Sequences alignment pointed that pig *Setd8a* and *Setd8b* variants shared the similarity with the predicted *Sus scrofa Setd8* sequence (XM_013982772.1). They all included the SET domain (Fig. 2).

Expression profiles of pig Setd8 variants

The RT-PCR results showed that the *Setd8* variants could be identified in testis of all ten male pigs. Histological staining was used to detect the structure of 180-day-old GZB pigs. As shown in Fig. 3, the elongated spermatid cells could be seen in the seminiferous tubules (Fig. 3). Then the GZB pigs in this study were called "puberty". The mRNA expressions of both *Setd8* variants in puberty GZB pigs were highly expressed in testis and brain tissues (Fig. 4, Fig. 5). Statistics analyses showed that the expressions of *Setd8a* and *Setd8b* in testis were significantly higher than in brain of puberty GZB pigs ($P < 0.05$) (Fig. 5). In addition, the expression levels of *Setd8a* were similar with *Setd8b* in testis of puberty GZB pigs (Fig. 5). Moreover, there was no or rare expression in heart, spleen, lung, kidney and liver of puberty GZB pigs. Then the function of *Setd8* variants in testis was worth to study.

Then the expression of *Setd8* variants in different periods of male pig testis was detected. As shown in Fig. 6, the expression levels of *Setd8* variants were both increased along with age, and the expression of two variants both reached the highest levels in puberty than other childhood periods ($P < 0.05$). When focused on 7-day-old and 30-day-old pigs, the expression level of

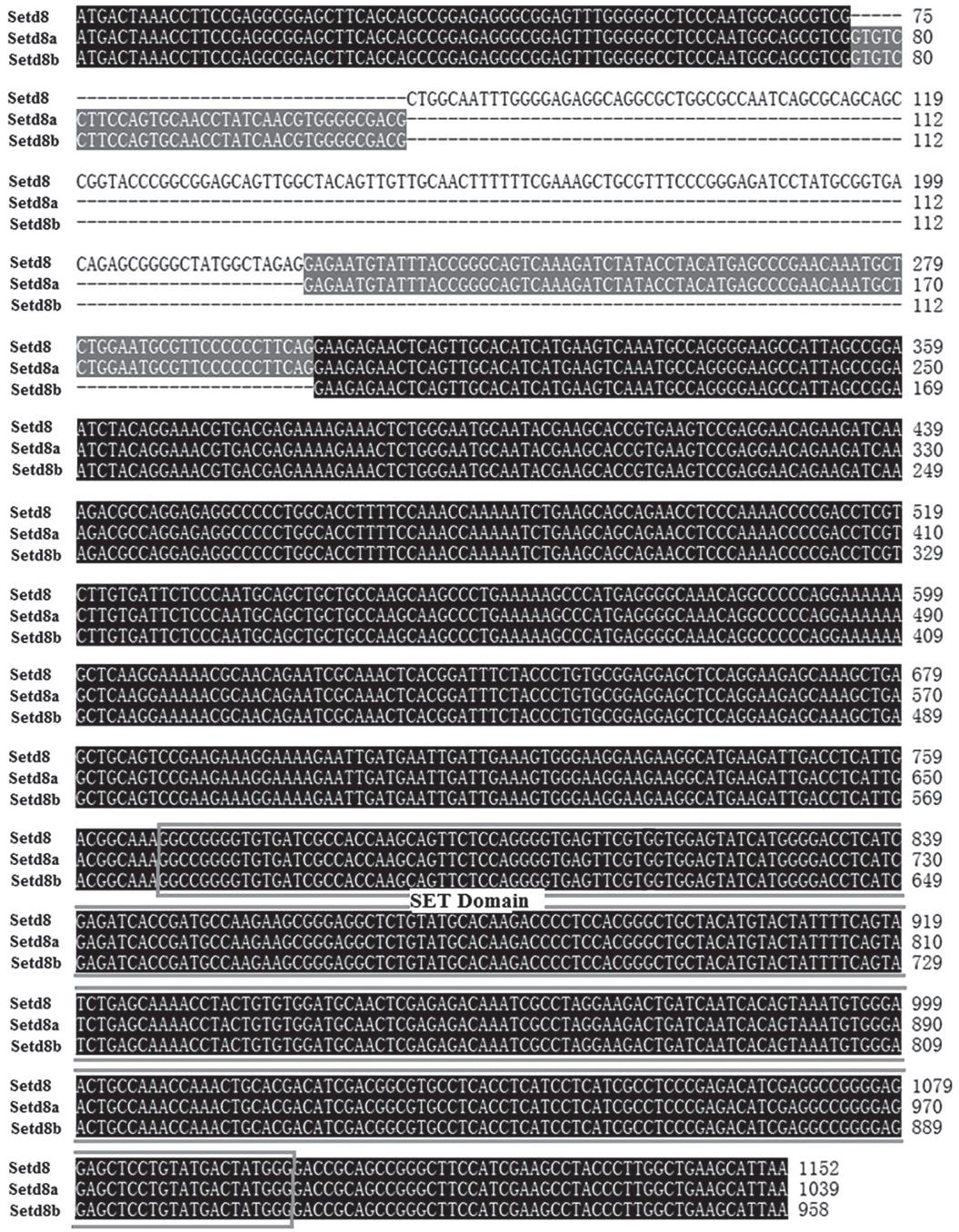

Fig. 2. DNA sequence alignments of *Setd8*, *Setd8a* and *Setd8b* in pig. Note: Setd8: predicted *Sus scrofa Setd8* mRNA (XM_013982772.1); Setd8a: *Sus scrofa Setd8* transcript variant *Setd8a* mRNA (KX254435); and *Setd8b*: *Sus scrofa Setd8* transcript variant *Setd8b* mRNA (KR021363). *Setd8a* and *Setd8b* were identified in this study. Gray and black shades indicated regions of two and three sequences identity, respectively. The red box showed the region of SET domain.

Setd8b was higher than that of *Setd8a* in testis ($P < 0.01$) (Fig. 6). However, as mentioned above, there was no significant difference in expression levels of *Setd8a* and *Setd8b* in testis of puberty GZB pigs.

Discussion

Published literatures had revealed that AS was a universal phenomenon in animals. As we all know, exons were confirmed with three major points: the 5' splice site, the 3' splice site and the branch point, which was called consensus "GT-AG" splicing rule[18]. Based on this condition, the mechanism of forming alternative splicing was simply summarized: exon skipping, intron retention, alternative 3' splice site and alternative 5' splice site[23,47]. AS can not only generate the transcript and protein diversity, but also influence cell differentiation and cell death[2,19,38]. The epigenetic regulators, such as Setd8, could occur the AS in mammalian, suggesting that the AS may be a driving force in regulating the function of these enzymes[21,27,28].

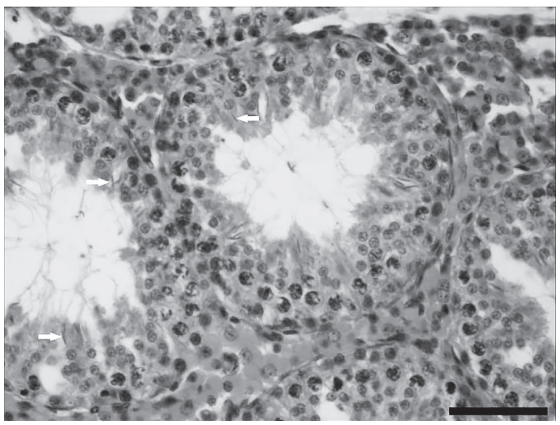

Fig. 3. H&E staining in 180-day old GZB pig testis (Scale bar = 50 μm). The emergence of elongated spermatid cells illustrated that the 180-day-old GZB pigs were puberty. Note: the white arrowheads indicated cell nucleus of elongated spermatid cells in pig seminiferous tubules.

Previous study demonstrated that *Setd8* was functionally essential for high efficient repair of DNA double strand breaks[5,9]. *Setd8* also played a role in human carcinogenesis by aberrant lysine methylation of PCNA, and it was essential for

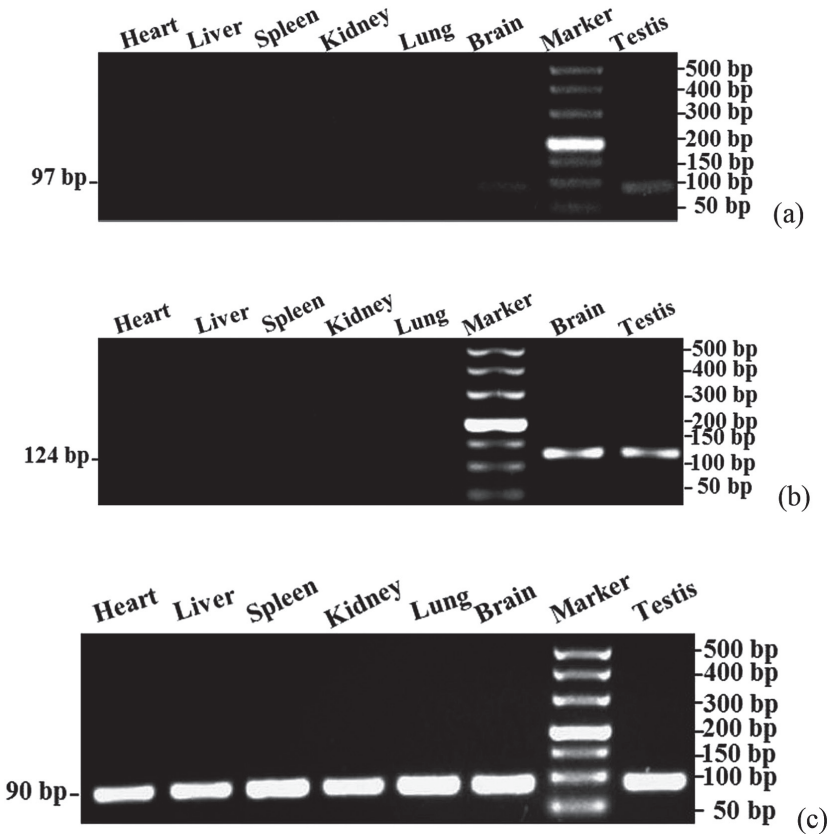

Fig. 4. The mRNA expression patterns of *Setd8a* (a), *Setd8b* (b) and *GAPDH* (c) in different tissues of 180-day-old GZB pigs.

genomic stability[15,41]. Moreover, *Setd8* was a context-dependent GATA-1 corepressor in erythroid cells, thus it was crucial in cell survival and maturation[4,29]. While deficiency of *Setd8* in c-Myc-overexpressing skin blocked cell proliferation and differentiation as well as caused apoptosis[7]. *Setd8* played a vital role in cell cycle, chromatin modification and organization.

The importance of *Setd8* in life made it important so that its structure should be further

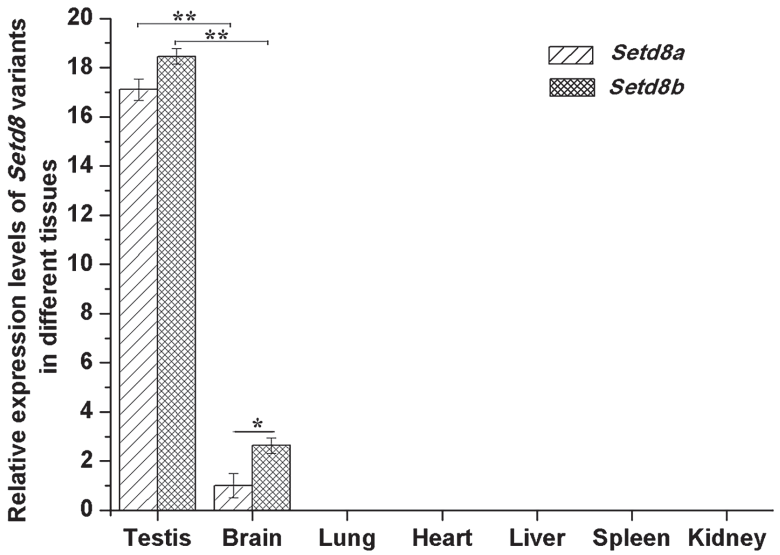

Fig. 5. Relative expressions of *Setd8a* and *Setd8b* variants in different tissues of 180-day-old GZB pigs. Note: The *GAPDH* was the housekeeping gene, and the mRNA expression levels of *Setd8a* and *Setd8b* were normalized to that of *GAPDH*. * and ** represented the significant difference ($P < 0.05$) and ($P < 0.01$), respectively.

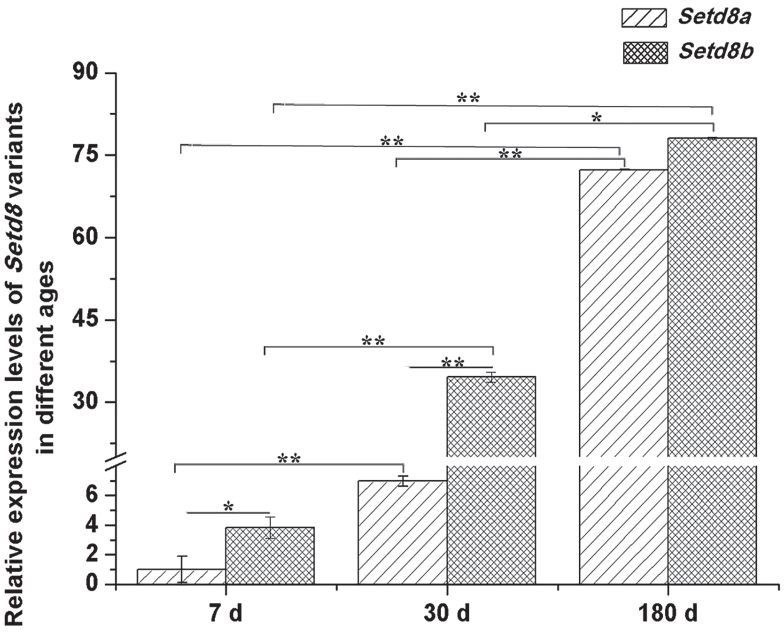

Fig. 6. Relative expressions of *Setd8a* and *Setd8b* variants in different periods of testis in male pigs. Note: The *GAPDH* was the housekeeping gene, and the mRNA expression levels of *Setd8a* and *Setd8b* were normalized to that of *GAPDH*. * and ** represented the significant difference ($P < 0.05$) and ($P < 0.01$), respectively.

examined. *Setd8* was firstly discovered and purified in Hela cells in 2002, while rare studies investigated its AS and mRNA expression in pig[31]. In this study, two variants were identified in testis and brain for the pig *Setd8* gene. Two variants, which named *Setd8a* and *Setd8b*, were found in human *Setd8* gene[1,10]. Sequence alignment of pig *Setd8a* and *Setd8b* indicated that they were similar to their counter-parts of human. Both of them had PIP box2 and SET domain. In addition, sequence analyses showed that the *Setd8* variants of human and pig all experienced the exon skipping. Moreover, *Setd8b* was the main functional splicing variant in human, and the result of qRT-PCR in this study was consistent with that in human[1,10]. Therefore, we presumed that pig *Setd8a* and *Setd8b* should perform the similar expression patterns to human *Setd8a* and *Setd8b*.

There were several points different between pig and human *Setd8* variants. At first, unique additional intron retention and partly missing exon were detected in pig *Setd8* variants, which enhanced the variants complexity in this study. However, the mechanisms how they formed were unknown. In addition, the mRNA expression of pig normal *Setd8* (GenBank Accession number: XM_013982772.1) was not detected in pig, predicting that the expression of *Setd8* was lowly or rarely in GZB pigs. However, the expression of human *Setd8* gene could be identified in testis.

Compared with other histone methyltransferases, the expression of *Setd8* was low somehow in tissues. Its abnormal expression could be contributed to human cancers[30]. Recent study showed that *Setd8* played an important role in erythroid survival, and deficiency of *Setd8* resulted in loss of H4K20me1[29]. These results predicted that *Setd8* would be expressed in blood, which didn't prove in this study. It was predicted by the expression of *Setd8* variants in the two tissues that *Setd8* might be benefit for reproduction of pigs.

It is said that 76% of all genes were expressed in brain and AS was related with different developmental stages[47]. In this study, *Setd8* variants were detected in brain and testis of puberty GZB pigs. Testis and brain were the most important organs regulating reproduction traits in pig. The result predicted that *Setd8* might play vital roles in brain, such as regulation of brain development. In addition, the expressions of *Setd8* variants were lower in brain than in testis. There were several studies showed that AS took an important role in species evolution and AS was always found in testis and cancer cell lines[16,18]. AS occurred in testis suggested a natural increase in the variation in expression to allow for evolutionary selection. Then the expressions of the two *Setd8* variants in testis were focused in this study.

In this study, three developmental stages (7-, 30 and 180-day-old) of pig testes were obtained to study the expression patterns of *Setd8* variants in testis. The germ cells were gonocytes in 7 days postnatal pig testis[48]. In addition, the gonocytes were developed into SSCs during 1~2 months in pig[48]. Then the SSCs differentiated in sperm to transferring genetic information to the next generation when pig reached a puberty age[48]. qRT-PCR results predicted the expressions of *Setd8* variants were time-regulated, and they might be related with the differentiation of germ cells. Moreover, *Setd8b* were mainly expressed in testis of young piglets, showed that *Setd8b* would be benefit to testicular development.

Conclusions

To summarize, two variants (*Setd8a* and *Setd8b*) were firstly identified in pig in this study. In addition, the two variants were expressed in testis and brain of puberty GZB pigs and the expression in testis was significantly higher than in brain. Moreover, the expressions of *Setd8* variants were time-dependent in different age pigs. It could be predicted that *Setd8b* was the major splicing variant of porcine *Setd8* transcript products, and *Setd8a* might play an important

role in testis and brain of puberty pigs.

Acknowledgments

This work was supported by Fundamental Research Funds for the Central Universities (NWSUAF, No.2452015145), China Postdoctoral Science Foundation funded project (No.2014M560809), Yangling Innovation Major Project on the Integration of Industry, Education and Research (2016CXY-17) and the National Basic Research Program of China (973 program; No.2014CB943100).

Compliance with ethical standards

Conflicts of interest

The authors declare no conflict of interest.

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