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Promoter DNA methylation and expression analysis of PIWIL1 gene in purebred and crossbred cattle bulls

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ABSTRACT

Major credit for India being the largest producer of milk in the world, goes to crossbred cows produced by inseminating low-producing indigenous cattle with semen from high producing exotic bulls. However, over the years, the policy of crossbreeding has been confronted with a major problem of subfertility in crossbred male progenies, culminating into disposal of a major fraction of mature bulls. Many studies have demonstrated relationship between epigenetic alterations and male fertility across different species. PIWIL1 is an important candidate gene for spermatogenesis and germ line development. Negative correlation between DNA methylation and expression of this gene has been highlighted in inter species hybrids of cattle and yaks. The present study envisaged elucidating promoter methylation status and expression profile of PIWIL1 gene in exotic Holstein Friesian cattle, indigenous Sahiwal cattle and their crossbreds with varying semen motility parameters. Semen samples were collected from bulls for isolation of DNA and RNA from spermatozoa. Bisulfite converted DNA was used to amplify promoter of PIWIL1 gene using methylation specific primers. The amplified products were sequenced after cloning in pTZ57R/T vector. The degree of methylation of the PIWIL1 promoter region was significantly higher in poor motility crossbred bulls (7.17%) as compared to good motility crossbreds (1.02%), Sahiwal (1.02%) and Holstein Friesian bulls (0.77%). PIWIL1 expression was 1.75, 1.71 and 1.59 folds higher in HF, Sahiwal and good motility crossbreds, respectively as compared to poor motility crossbreds.

Key words: Cattle, Crossbreeding, Expression, Fertility, Methylation, PIWIL1

India has registered a massive milk production of 163.7 million tonnes during 2016–17 with a remarkable growth rate of 20.13% as compared to 2013–14. It is imperative for a country like India to maintain a progressive trend in milk production to meet the nutritional need of the 17.74% of the total world population that it possesses. The contribution of crossbred cattle (26%) to total milk production is 2–3 folds higher as compared to indigenous (12%) and non-descript cattle (9%) (DADF Annual Report 2017, www.dahd.nic.in). Substantial heterosis in terms of increase in lactation milk yields, lactation lengths, and decrease in calving interval and age at first calving ensured that crossbreeding remains an important breeding policy in India (Galukande *et al.* 2013). However, over the years, decline in genetic diversity of indigenous germplasm and deterioration in vital traits like viability, disease resistance and reproduction have surfaced as the ill effects of crossbreeding. Most importantly, subfertility of crossbred male progenies in terms of poor seminal parameters results in disposal of a major proportion of mature bulls (Ahlawat *et al.* 2017).

Spermatogenesis, a complex process, requires tight spatial and temporal gene regulation for production of mature spermatozoa from undifferentiated germ cells (Bak *et al.* 2011). Gene expression is regulated by epigenetic modifications such as DNA methylation that establish heritable cellular memories. The dynamism and tissue specificity of the epigenetic code has generated great interest in epigenetic control of key physiological processes (De Carvalho *et al.* 2010). Many studies highlighted relationship between epigenetic alterations and male subfertility in humans (Urdinguio *et al.* 2015, Laqqan *et al.* 2017). Even in bovines, negative correlation between DNA methylation and gene expression for reproduction related genes, was reported. Sterile males produced by crossing cattle (*Bos taurus*) and yak (*Bos grunniens*) were extensively investigated from this perspective (Yao *et al.* 2015). In light of these studies, epigenetics has emerged as a promising research field for deciphering the molecular aspects of male infertility/subfertility. Such studies are relevant to identify biomarkers for sire fertility, since traditional semen analysis (sperm count, motility and morphology) is crippled by significant intra- and inter-individual variation with respect to these parameters (Leushuis *et al.* 2010).

Small noncoding RNAs are regarded as important mediators of posttranscriptional regulation of gene expression during germ cell maturation. Among them,

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piRNAs (PIWI-interacting RNAs) are reported to be essential for germ cell maintenance in animals (Girard *et al.* 2006). They bind to their effectors, PIWI proteins, to ensure repression of selfish genetic elements known as transposons (Vagin *et al.* 2006). PIWI proteins belong to Argonaute protein family and include three members in Drosophila and murine genomes whereas four members in bovine, porcine, and human genomes (Russell *et al.* 2016). PIWI proteins associate with piRNAs to form PIWI-interacting RNA com-plexes (piRC) that in turn bind RNA targets. Subsequently, piRC triggers gene silencing by either cleavage of the target transcript or direct methylation-dependent transcriptional silencing toward target loci in the genome (Aravin *et al.* 2008). The importance of piRNA-PIWI complex in spermatogenesis is substantiated by extraction and purification of this complex from mammalian testis (Lau *et al.* 2006). Taken together, PIWI proteins have pivotal roles in male gametogenesis by ensuring protection of the differentiating germ cells' genomic stability and consequently, influence male fertility (Giebler *et al.* 2017).

Of particular interest is PIWIL1, which regulates the turnover of mRNAs and long noncoding RNAs during spermatogenesis (Gou *et al.* 2014). In model organisms like Drosophila and zebrafish, PIWIL1 homologues are required for both male and female fertility. Strikingly, the PIWIL1 homologue, MIWI in mice is crucial for male reproduction, but does not affect female fertility. Deletion of PIWIL1 leads to male infertility; but the females remain fertile, thus establishing its role in spermatogenesis (Russell *et al.* 2016). Gou *et al.* (2017) reported that single nucleotide mutations in PIWIL1 result into azoospermia in humans. An interesting study from bovines involves analysis of mRNA expression and DNA methylation patterns of PIWIL1 in the testes of cattle, yaks, and their sterile hybrids. Significantly higher methylation levels and lower gene expression was observed in the testes of cattle-yak crosses as compared to cattle or yaks (Gu *et al.* 2013). However, till date, there are no studies regarding genetic and epigenetic regulation of PIWIL1 in cattle and their crossbreds. Thus, the objective of the present study was to assess the promoter methylation status and expression profile of PIWIL1 gene in exotic Holstein Friesian (HF) cattle, indigenous Sahiwal cattle and their crossbreds with varying semen motility parameters.

MATERIALS AND METHODS

Collection of semen samples: Semen samples of Holstein Friesian (HF) and Sahiwal bulls with good sperm motility parameters were collected from Semen Bank, Hisar, Haryana. Crossbred bull (Holstein Friesian × Sahiwal) samples were obtained from ICAR-CIRC, Meerut. Samples were divided into 2 groups, viz. Gr I (good motility bulls) and Gr II (poor motility bulls) (Table 1). All experiments were approved and performed as per the guidelines of the Institute Animal Ethics Committee.

DNA isolation, bisulfite conversion and PCR: Lysis of spermatozoa was performed as per Sarova *et al.* (2018), followed by standard phenol chloroform method for extraction of DNA. Integrity of DNA was determined by electrophoresing the dissolved DNA on 0.8% agarose gel stained with ethidium-bromide (0.5 µg/ml) in 1× TAE buffer. Quality and quantity of extracted DNA was assessed from optical density values at 260 and 280 nm on Nanodrop spectrophotometer (ND-1000, Thermo Scientific). After determining the concentration and purity of DNA, it was diluted to a final concentration of 100–150 ng/µl in nuclease free water as working stock and stored for further use at 4°C. This was followed by bisulfite conversion of genomic DNA isolated from each semen sample with Invitrogen MethylCode™ Bisulfite Conversion Kit as per the manufacturer's recommendations. After bisulfite conversion, the samples of the four genetic groups (HF, Sahiwal, good motility and poor motility crossbreds) were pooled separately. Methylation profile of PIWIL1 gene promoter was assessed by producing an amplicon of 336 bp using the following methylation specific primers: AATTGTTTAGAGTGGATATTGAGG (Forward) and CCCACTAACAACAAAAAACAC (Reverse) (Gu *et al.* 2013).

Table 1. Details about cattle bulls included in the study

Category	Number of samples	Initial sperm motility	Post thaw motility
Crossbred (Poor motility)	5	40–60%	–
Crossbred (Good motility)	5	80–85%	55–60%
Sahiwal	5	80–85%	55–60%
Holstein Friesian	5	>85%	60–65%

Cloning and sequencing of PIWIL1 *promoter region:* Purification of the amplified PCR product was done using GenetixNucleo-pore® PCR clean-up gel extraction kit and the presence of purified product was confirmed by 2% agarose gel electrophoresis. Thermo Scientific InsTAclone PCR cloning kit was used for ligation of the purified PCR product with pTZ57R/T vector as per manufacturer's instructions. Transformation experiments were performed with DH5α strain of *E. coli* and the cells were made competent by giving calcium chloride treatment (Sambrook *et al.* 1989). After this, spreading of the culture was done on Luria Bertani agar plates containing 100 mg/ml ampicillin, 0.1 M IPTG and 20 mg/ml X-Gal. The LB agar plates were incubated at 37°C overnight, followed by selection of recombinant clones by blue-white screening. Isolation of plasmids carrying the cloned fragment was done using conventional method. Sequences for 15 independent clones from each pool were obtained using the ABI PRISM BigDye™ Terminator v3.1 Cycle Sequencing Kit protocol (Applied Biosystems, Foster City, CA) on an automated DNA sequencer (Applied Biosystems-3100 Avant). Sequence alignments and analysis were performed using multiple sequence alignment tool MAFFT (http://mafft.cbrc.jp/alignment/software/). Statistical analysis was done using SPSS v17.0 for Windows and significant differences were set at $P<0.05$.

RNA isolation, cDNA preparation and gene expression analysis: Isolation of total RNA from semen samples was done using TRIzol reagent (Sigma-Aldrich), followed by removal of traces of genomic DNA by on-column DNAse I digestion and finally, purification using RNeasy Kit (Qiagen, USA). Integrity of extracted RNA was ascertained by running 2 µl RNA sample on 1.5% agarose gel. Quantification of purified RNA was done using Nanodrop spectrophotometer (ND-1000, Thermo Scientific). SuperscriptIII reverse transcription system (Invitrogen) was used for first strand cDNA synthesis. For this, 200 nanogram of total RNA, 1 µL of 50 µM oligo (dT) and 1 µL of 10 mM dNTPs in a total volume of 13 µL, were incubated for 5 min at 65°C and immediately chilled on ice. This was followed by addition of 4 µL of 5× first strand buffer, 1 µL of DTT (0.1 M), 1 µL of RNaseOUT (40 units/µL) and 1 µL of Superscript reverse transcriptase III (200 units/µL). The reaction mixture was incubated at 42°C for 1 h and finally, at 70°C for 15 min. PIWIL1 transcript level across different genetic groups was calculated using the comparative cycle threshold method (Livak and Schmittgen 2001). House-keeping genes, i.e. Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) and β-actin were used for normalization of the data. The details of the primers used are given in Table 2.

Real-time PCR was performed on Light Cycler 480 System (Roche Diagnostics, USA) using SYBR Green I master mix. Reaction mix (20 µL) consisted of 2 µL cDNA, 0.4 µL of each primer (10 mM), 10 µL of 2× SYBR Green I master mix and appropriate volume of nuclease free water. The amplification cycle involved template denaturation at 95°C followed by two-step primer annealing, amplification and quantification program which included 45 cycles of 95°C for 10 sec, annealing at 60°C for 20 sec and finally cooling at 4°C. Melting curve analysis was used to determine the specificity of the amplified PCR products. Expression analysis was done for 5 biological and 2 technical replicates of each genetic group along with a non-template control for each gene. Crossbred bulls with poor motility were considered as control for calculating fold change across different groups. Analysis of the gene expression data was done using one way ANOVA followed by Tukey's multiple comparison tests.

RESULTS AND DISCUSSION

Genomic DNA was isolated from semen samples of 5 bulls each of Holstein Friesian, Sahiwal, good motility and poor motility crossbred category. Electrophoresis of isolated DNA samples on 0.8% agarose gel produced intact bands with no shearing revealing successful isolation of good quality DNA. Ratio of optical density values at 260 and 280 nm on Nanodrop spectrophotometer ranged from 1.75 to 1.8 indicating desirable DNA quality for further work. After dilution of stock DNA to a working concentration of 100–150 ng/µl, it was subjected to bisulfite conversion. Equal concentration of bisulfite-converted DNA of these samples was subsequently pooled into separate genetic groups. An amplicon of 336 bp covering part of PIWIL1 promoter spanning positions –3083 to –3418 with respect to NCBI accession number NC_037344.1 was successfully amplified using methylation specific primers from pooled DNA samples. The level of methylation of the 26 CpG sites in the PCR product was determined after cloning and sequencing the amplified products. Methylation profile of the CpG sites of the PIWIL1 promoter in different genetic groups is depicted in Fig. 1. Bisulfite sequencing revealed that the degree of methylation of CpG sites in the amplified fragment was very low in all the samples. Nevertheless, the degree of methylation was significantly higher in poor motility crossbred bulls (7.17%) as compared to good motility crossbreds (1.02%), Sahiwal (1.02%) and Holstein Friesian bulls (0.77%) ($P<0.05$). Out of 26 CpG sites amplified, methylation marks were evident at 15 sites in poor motility crossbreds and only 2 sites in the other three groups. Extremely low methylation level of PIWIL1 promoter was also observed in mice spermatozoa, which lends support to our observations (Borgel *et al.* 2010).

Real Time-PCR was used to determine transcript levels of PIWIL1 gene in different genetic groups. The extracted RNA from individual samples exhibited high purity as revealed by mean (±SEM) $A_{260/280}$ ratio of 2.02±0.01. Specificity of the reaction and absence of primer-dimers or nonspecific products was confirmed by melting curve analysis of the amplified PCR products. The geometric mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin was used for normalization of data. Poor motility crossbred bulls were taken as control for estimating fold change of PIWIL1 across different groups. Expression was 1.75, 1.71 and 1.59 folds higher in HF, Sahiwal and good motility crossbreds, respectively as compared to control ($P<0.01$).

Many researchers over the years have attempted to

Table 2. Primers used for gene expression analysis by qRT-PCR

Gene	Primer sequence	Amplicon size	Annealing temperature	Reference
PIWIL1	F: GGCAGTGAGAAGTGGTAGT R: AGGAGCAGGAACACGAATG	148 bp	60°C	Gu *et al.* (2013)
GAPDH	F: TGGAAAGGCCATCACCATCT R: CCCACTTGATGTTGGCAG	60 bp	60°C	Kapila *et al.* (2013)
β-actin	F: GCGTGGCTACAGCTTCACC R: TTGATGTCACGGACGATTTC	56 bp	60°C	Kapila *et al.* (2013)

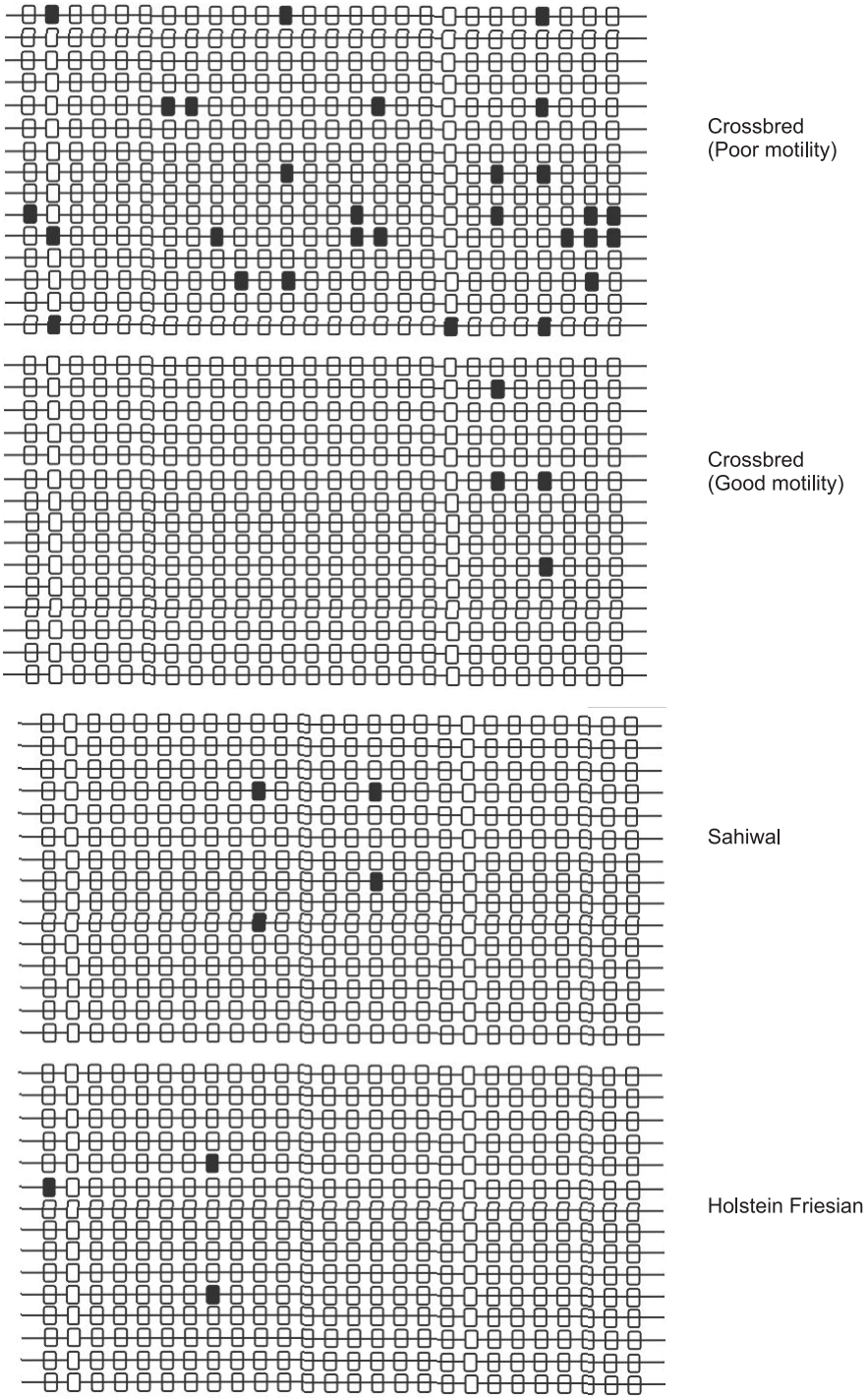

Fig. 1. DNA methylation status of the PIWIL1 promoter in different groups. Each row represents the sequence of an individual clone. Black and white squares indicate CpG methylated and unmethylated sites, respectively.

understand the mechanism of interspecific hybridization from a DNA methylation perspective. Koroma *et al.* (2011) analyzed methylation patterns of interspecific Xenopus F1 hybrids and reported higher proportion of methylated fragments in hybrids as compared to the parental species. Another intriguing observation was higher methylation levels in sterile hybrid males than infertile hybrid females. Cattle-yak hybrids being crosses of *Bos taurus* and *Bos grunniens* and crossbred cattle being crosses of *Bos taurus* and *Bos indicus* are also examples of interspecific hybridization. In order to investigate the role of PIWIL1 gene in male sterility of cattle-yak hybrids, Gu *et al.* (2013) analyzed mRNA expression and DNA methylation patterns of this gene in the testes of cattle, yaks, and their crosses. They observed significantly higher methylation levels of the PIWIL1 gene in cattle-yak hybrids as compared to cattle

or yaks. The methylation profile was in concordance with gene expression results, since PIWIL1 transcript level was significantly lower in the crossbreds as compared to the parents. They speculated that these differences could affect transposon silencing mechanism of PIWIL1, thus contributing to cattle-yak male sterility. Our results and observations of Guv *et al.* (2013) are in line with the findings of Koroma *et al*. (2011), since more methylation was apparent in subfertile cattle crossbreds and sterile cattle yak hybrids. Hence, studies that generate strong support for DNA methylation as a contributing factor for interspecific hybridization become relevant in understanding the concept of reproductive isolation. Interestingly, PIWIL1 mouse mutants exhibit sterility in males whereas fertility in females (Russell *et al.* 2016). This makes PIWIL1 a prospective candidate gene for male fertility.

Reports about inverse correlation between promoter methylation and PIWIL1 expression are also available from poultry (Qiu *et al.* 2016). Strong evidence was provided for methylation-mediated transcription factor regulation in controlling spatiotemporal expression of chicken PIWIL1 from the primordial germ cell stage to the round spermatid stage. An attempt to decipher the epigenetic basis of idiopathic male infertility using next-generation sequencing of spermatozoa RNAs in humans delineated prospective candidate genes central to spermatozoa competence, one of which was identified to be PIWIL1 (Pereira *et al.* 2016). Another significant study quantifying expression of PIWIL proteins (PIWIL1 and *PIWIL2*) in human ejaculated spermatozoa showed significant correlation of mRNA expression with semen parameters such as sperm count and progressive motility (Giebler *et al.* 2016).

Studies are also underway regarding global genomic DNA methylation profiles in relation to fertility. For instance, a disease-associated DNA methylation profile, characterized by targeting members of the PIWI-associated RNA (piRNA) processing machinery was identified in human infertile male patients with spermatogenic disorders by using the Infinium Human Methylation 27 BeadChip. Male infertility was reported to be associated with the promoter hypermethylation-associated silencing of piRNA-processing genes, particularly PIWIL1 and *PIWIL2* (Heyn *et al.* 2012). A possible relationship between SNPs in piRNA pathway genes and male infertility has also been explored. An SNP in the 3' UTR (rs508485T>C) of human PIWI gene (PIWIL4) was significantly associated with increased oligozoospermia risk in Han-Chinese population (Gu *et al.* 2010). Two allele-specific methylation-sensitive SNPs in PIWIL1 (rs10773767) and PIWIL2 (rs6982089) have also been identified using an array-based study performed with peripheral blood samples from 30 infertile men (Friemel *et al.* 2014). Epigenetic aberrations in the activity of PIWI proteins and piRNAs have been associated with testicular cancers. Promoter CpG island hypermethylation associated silencing of PIWIL1, *PIWIL2*, *PIWIL4* and *TDRD1* was observed in primary seminoma and non-seminoma testicular tumors (Ferreira *et al.* 2014). All these studies strengthen the evidence to support the importance of DNA methylation differences in piRNA pathway genes with impaired spermatogenesis.

Many reports from cattle, yaks and their hybrids have provided considerable insights into genetic and epigenetic control of fertility. Simultaneous profiling of gene expression and promoter methylation status of many spermatogenesis related genes (*Boule*, *BvH, DAZL* and *Mei1*) has yielded promising results. For all these genes, promoter DNA was hypermethylated in the testes of cattle-yak hybrids as compared to parents. Coinciding with the dramatic increase in methylation level, expression of these meiosis specific genes was reported to be lower in the hybrids (Yao *et al.* 2015). These studies lend support to the concept of negative relationship between methylation levels and transcript abundance of genes. Sarova *et al.* (2018) analyzed the methylation profile of *DAZL* gene in spermatozoa of purebred and crossbred cattle bulls. Significantly higher methylation of CpG sites was evident in Frieswal bulls with poor motility (28.26%) as compared to good motility bulls (6.52%). Interestingly, indigenous cattle breed, Sahiwal showed intermediate level of methylation (15.21%), but the exotic Holstein Friesian animals had least values (4.34%). Negative correlation between promoter methylation and gene expression of *Bvh* gene was also observed in spermatozoa from crossbred bulls with poor motility phenotype as compared to purebred parents (Ahlawat *et al.* 2018). Methylation levels across *IGF2-H19* differentially methylated region (DMR) locus have been assessed in spermatozoa from Karan Fries bulls (Holstein Friesian × Tharparkar) categorized into high and low fertility groups based on conception rates. The degree of methylation at *IGF2-H19* DMRs did not vary among the two categories. Nevertheless, methylation difference in the sixth CTCF binding site (CCCTC) between the two groups was speculated to account for graded fertility status of crossbred bulls (Jena *et al.* 2014).

This study observed hypomethylation of PIWIL1 gene promoter in spermatozoa of cattle bulls. Nonetheless, the epigenetics marks were more in poor motility crossbred bulls as compared to good motility crossbreds, indigenous Sahiwal and exotic Holstein Friesian bulls. The gene expression results were in concordance with methylation status since transcript level of PIWIL1 was significantly lower in poor motility crossbreds relative to all other genetic groups. Our findings are consistent with previous findings of negative correlation between DNA methylation and gene expression in different species such as chickens, humans, cattle and yaks. To the best of our knowledge, this is the first report in cattle in which genetic and epigenetic control of PIWIL1 gene has been investigated in bull spermatozoa to unveil the molecular basis of subfertility in crossbred bulls.

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