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Whole-genome bisulfite sequencing of X and Y sperm in Holstein bulls reveals differences in autosomal methylation status

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Abstract

A comprehensive understanding of the molecular differences between X and Y sperm in Holstein bull semen is crucial for advancing sex control technologies. While previous studies have primarily focused on proteomic and transcriptomic differences, the genome-wide DNA methylation differences between these sperm types remains largely unexplored. In this study, we employed whole-genome bisulfite sequencing to systematically compare the autosomal methylation profiles of X and Y sperm. Although global methylation patterns showed remarkable consistency between the two sperm types, our localized comparative analysis revealed 12,175 differentially methylated regions mapping to 2,041 genes (differentially methylated genes, DMGs). Functional enrichment analysis of these DMGs revealed their involvement in essential biological processes, particularly in energy metabolism and membrane voltage regulation. Notably, *SPA17* and *CHCHD3*, identified as hypermethylated genes in X sperm in this study, have also been reported to show lower protein expression levels in X sperm compared to Y sperm. Furthermore, we identified 28 DMGs functionally associated with spermatogenesis and 5 DMGs related to fertilization. Our findings lay the foundation for thorough understanding of molecular differences between X and Y sperm in bull, providing essential insights for the development of more advanced sex control technologies in the future.

Keywords Holstein bull, Whole-genome bisulfite sequencing, DNA methylation, X sperm and Y sperm, Epigenetics

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Introduction

Controlling the sex ratio of Holstein cattle to favor female offspring for milk production is crucial for improving farm profitability. Currently, flow cytometric sorting of X and Y sperm is the primary commercial method for sex control in cattle, leveraging the approximate 3.8% difference in DNA content between X- and Y-bearing sperm. However, this method has certain limitations, including high costs and slow sorting rates. Additionally, some studies have reported reduced fertilization rates and compromised embryo development following insemination with sex-sorted semen [1, 2]. While advancements in this technology have significantly improved its efficiency and made it widely applicable in artificial insemination, particularly in North American dairy herds, the development of new sex control strategies remains an important area of research. Further exploration of molecular and functional differences between X and Y sperm could provide valuable insights for developing alternative and potentially more effective sex control techniques.

Epigenetics is a dynamic and reversible process that plays a crucial role in establishing and maintaining normal cellular functions [3, 4]. Compared to other epigenetic regulatory elements, such as histone acetyltransferases and chromatin remodeling enzymes, DNA methylation is the most biologically stable epigenetic mechanism [5]. Numerous studies have demonstrated that DNA methylation is closely linked to sperm quality and spermatogenesis [6–8]. Semen quality parameters, including total sperm count, motility, and morphological abnormality rate, are all influenced by DNA methylation [9–11]. Notably, aberrant methylation at 9,189 CpG sites (CGs) has been associated with reduced sperm motility, with 80% of these sites exhibiting lower methylation levels [10]. The relationship between DNA methylation at imprinted genes and sperm quality has also attracted considerable attention [12, 13]. Specifically, the loss of methylation at *H19* and the gain of methylation at *GTL2* and *MEST* have been linked to an increased incidence of azoospermia and oligospermia [13]. Beyond *H19*, decreased methylation levels in imprinted genes such as *LIT1*, *MEST*, *SNRPN*, *PLAGL1*, and *PEG*3 have been implicated in reduced sperm counts and sperm maturation disorders [12].

For decades, researchers have been striving to identify substantial differences between X and Y sperm across various aspects, including morphology, motility, pH, stress response, and electric charge [14, 15]. However, the existence and extent of these differences remain controversial. Nonetheless, multiple proteomic and transcriptomic studies have identified several proteins and genes that are differentially expressed between X and Y sperm [16–19]. Regarding DNA methylation, most studies have focused on sex differences in certain tissues of humans and pigs, as well as in specific cell types such as blood cells, cardiac muscle, liver, and pancreatic tissue [20, 21]. In humans, DNA methylation in whole blood exhibits sex-specific patterns, with males generally showing higher methylation levels at repetitive elements, including ALU and LINE-1 [22]. Another study investigating sex differences in DNA methylation within human pancreatic islets found no significant global methylation differences between sexes on autosomes, whereas X-chromosome methylation was higher in female islets compared to male islets [23].

However, to our knowledge, studies on DNA methylation differences between X and Y sperm in livestock are still limited. Thus, this study aims to understand the differences in the autosomal methylation profiles between these sperm types and identify important differentially methylated genes (DMGs) related to sperm epigenetics, in bulls. Our study provides a comprehensive resource for bovine epigenomic research and offer new insights into DNA methylation differences between X and Y sperm, potentially identifying specific markers for X and Y sperm sorting.

Materials and methods

Sperm collection and DNA extraction

We collected semen samples from three fertile, healthy Holstein bulls (ID 291HO16057, 291HO17050, 291HO17064) at the Saikexing Institute (Inner Mongolia SaiKeXing Institute of Breeding and Reproductive Biotechnology in Domestic Animals, Hohhot, China), with further details available on their official website (http://www.saikexing.com/seedBullDataindex.action?cid=1%26sid=6). The semen samples were collected using an artificial vagina and sorted as described in a previous publication [19]. Briefly, the samples were stained with Hoechst-33,342 fluorophore (Sigma, St Louis, USA), and then separated into X and Y sperm using a high-speed MoFlo SX XDP flow cytometer (DakoCytomation, Fort Collins, USA). The purity of the X and Y sperm samples was assessed using the sort reanalysis method [24]. As a result, we obtained over 120 million sperm for each type, with purity above 90%.

Genomic DNA was extracted using the Sperm DNA Purification Kit (Simgen, Hangzhou, China) according to the manufacturer's instructions. The quantity and quality of the extracted sperm DNA were assessed using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and DNA agarose gel electrophoresis. The genomic DNA from all samples was then used to construct whole-genome bisulfite sequencing (WGBS) libraries.

WGBS library construction and sequencing

Briefly, 3 μg of genomic DNA, spiked with unmethylated lambda DNA, was fragmented into 200–300 bp

fragments using a Covaris S220, followed by terminal repair and A ligation. Different cytosine methylated barcodes were ligated to sonicated DNA from different samples. Bisulfite conversion of DNA was performed using the EZDNA Methylation Gold Kit (Zymo Research). Single-stranded DNA fragments were then amplified using the KAPA HiFi HotStart Uracil + ReadyMix (2×) (Kapa Biosystems, Wilmington, MA, USA). The library concentration was quantified using a Qubit 2.0 fluorometer and qPCR (iCycler, BioRad Laboratories, Hercules, CA, USA), and the insert size was verified using the Agilent 2100. To reduce batch effects, libraries for each sample were balanced and mixed with libraries from other samples with different barcodes, and sequenced on separate lanes of a HiSeq X Ten platform to generate 150-bp paired-end reads by Novogene (Novogene, Beijing, China).

Raw data profiling and methylation calling

We used FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trim Galore v0.4.0 (https://github.com/FelixKrueger/TrimGalore) to assess the quality of the sequencing data. Adapters were removed, and reads with low quality (Q < 20) or shorter than 20 bp were filtered out. The cleaned reads were aligned to the reference genome ARS-UCD1.2 for bull sperm using Bowtie2 [25]. After removing duplicate reads, we employed Bismark [26] to extract methylcytosine information.

Global comparison between methylomes of X and Y sperm

The common CGs with a depth greater than 10 × among all samples were used for global comparison between each of the two sample pairs. Detection of differentially methylated regions (DMRs) were applied using an R package (methylKit, R version 3.3.3) [27]. Specifically, for the global comparison of DNA methylation levels, we applied the Fisher exact test to assess the DNA methylation levels of 1000 bp windows across the entire cattle genome using the methylKit software. Within each 1000 bp window, the average methylation level was calculated by averaging the methylation levels of all CGs within the window. After calculating *P* values for differential methylation, we used the SLIM method [28] for multiple testing correction to obtain *Q* values.

DMRs were defined as regions with an average methylation difference greater than 25% and a *Q* value < 0.05. Genome structure annotation files for genes and repeat elements were downloaded from the NCBI database (ARS-UCD1.2) [29]. In this study, promoter regions were defined as the 1000-bp upstream and downstream regions flanking transcription start sites.

Clustering of samples

Samples were clustered based on the similarity of their methylation profiles. The clustering analysis was performed using the clusterSamples function of methylKit, which calculates pairwise distances between samples based on their methylation profiles and applies hierarchical clustering. The distance metric was set to "correlation", and the clustering method was set to "ward". A dendrogram was generated to visualize the clustering results.

Functional enrichment analysis of DMGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on DMGs using the DAVID online tool (https://davidbioinformatics.nih.gov/) [30]. Gene enrichment within annotation terms was assessed using Fisher's exact test, and *P* values were adjusted using the false discovery rate (FDR) method to identify significantly enriched terms.

Results

Methylomes of X sperm and Y sperm in bulls

We generated single-nucleotide resolution methylation profiles for X and Y sperm from bulls using WGBS. Each type of sperm was separately collected from the same three bulls as biological replicates. In total, we obtained an average of 630,462,105 clean reads per sample, with an average mapping rate of 69.75% (Table 1). Successfully mapped reads provided an average genome coverage of at least 21× for each sample. Consistent with our previous studies [31], methylation was predominantly observed in the CG context, with an average methylation level ranging from 71.70 to 77.40%. In contrast, non-CG contexts (CHH and CHG, where H = A, C, or T) were rarely methylated, with an average methylation level below 1%.

Table 1 Details of the methylation sequencing data

Sample	50X	50Y	57X	57Y	64X	64Y
Clean reads	643,727,954	651,667,916	646,674,336	592,169,732	577,268,174	671,264,520
Sequencing depth	~24 ×	~25 ×	~24 ×	~21 ×	~21 ×	~25 ×
Mapping efficiency	70.3%	69.5%	71.2%	68.2%	70.0%	69.3%
BS conversion	99.38%	99.39%	99.36%	99.33%	99.35%	99.40%
C methylated in CpG	77.40%	75.70%	75.80%	71.70%	72.70%	73.60%
C methylated in CHG	0.50%	0.50%	0.70%	0.70%	0.70%	0.70%
C methylated in CHH	0.40%	0.50%	0.70%	0.80%	0.70%	0.70%

Methylation patterns were highly conserved across sperm samples, with correlation coefficients exceeding 0.9 between sample pairs (Fig. 1A). Repetitive sequences in sperm, including long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), long terminal repeats (LTRs), and DNA transposons, exhibited high methylation levels (Fig. 1B), consistent with methylation patterns observed in humans [6, 32]. High methylation is known to suppress transposon activity, primarily by inhibiting its transcription [33, 34]. However, satellite repeats, which are typically highly methylated in somatic cells, exhibited low methylation levels in sperm, consistent with previous reports [32, 35]. Within gene regions, promoter methylation was below 20%, while introns displayed higher methylation levels than exons. CpG islands (CGIs) also showed relatively low methylation levels, averaging below 20%. Furthermore, by analyzing the average methylation levels of CGIs and promoters, both genomic features exhibited a distinct bimodal distribution pattern (Figure S1).

Identification of DMRs between X and Y sperm

In the correlation analysis, we did not observe a higher correlation within X or Y sperm samples. Instead, the X and Y sperm from the same individual exhibited the highest correlation (Fig. 1A). This was further supported by cluster analysis, which showed that X and Y sperm clustered according to their respective bulls, rather than being separated by sperm types (Fig. 2A). These findings suggest that the methylation differences between X and Y sperm may be relatively small.

In total, we identified 12,175 DMRs between X and Y sperm, including 5,967 hypomethylated DMRs and 6,208 hypermethylated DMRs in X sperm compared to Y sperm. In our previous study [31], we successfully classified computationally annotated CpG islands (cCGIs) into two categories: eCG islands (eCGIs, experimentally supported CGIs) and neCG islands (neCGIs, cCGIs that do not overlap with eCGIs). Compared with other genomic features, we found that the eCGIs and promoter were the primary genomic features enriched with DMRs (Fig. 2B). The DNA methylation status of these regions may have affected gene expression, which was consistent with previously reported differences in gene expression between X and Y sperm [16, 17].

Functional enrichment analysis of genes overlapped with DMRs

We identified 1,050 genes overlapping with hypomethylated DMRs and 991 genes overlapping with hypermethylated DMRs in X sperm compared to Y sperm. GO enrichment analysis showed that hypermethylated genes were mainly associated with GO terms such as GABA-A receptor complex, cellular response to cAMP, and cytosol (Fig. 3A). In contrast, hypomethylated genes were primarily enriched in GO terms related to ATP binding, extracellular-glutamate-gated ion channel activity, and Golgi apparatus (Fig. 3B).

Moreover, KEGG enrichment analysis showed that hypermethylated genes were significantly enriched in the cAMP signaling pathway, GABAergic synapse, and nicotine addiction (Fig. 4A). Significant enrichments were also found in biological processes related to oxytocin signaling pathway, long-term depression, and dopaminergic synapse when analyzing hypomethylated genes (Fig. 4B).

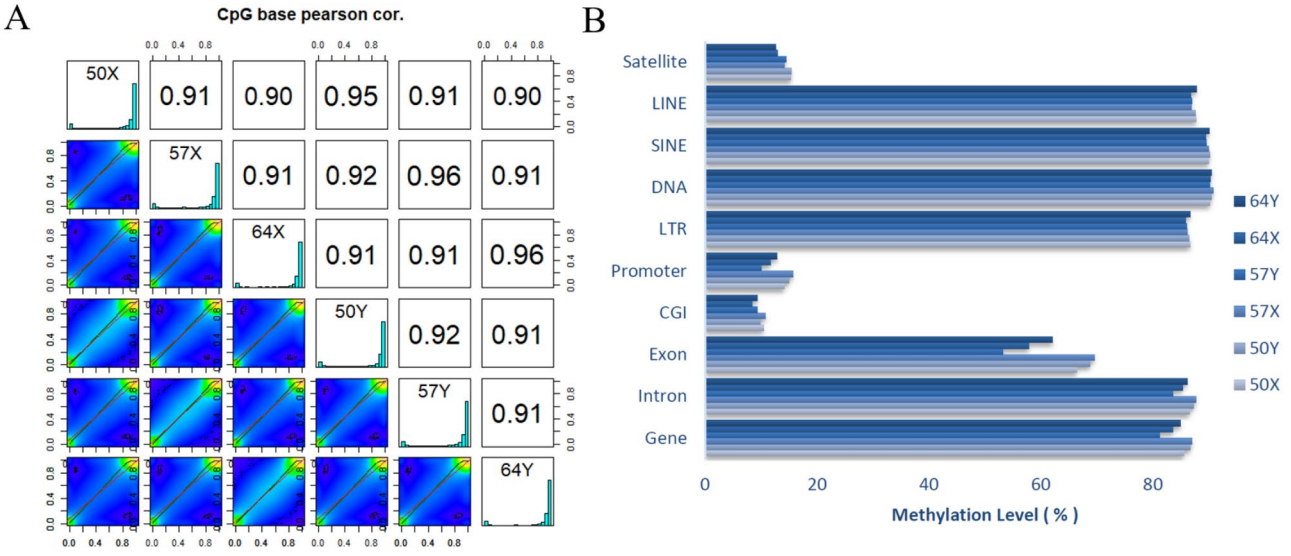

Fig. 1 Genomic distribution of methylated loci in bull sperm. (**A**) Correlation of DNA methylation between X and Y sperm from three Holsteins, numbered 50, 57, and 64. The intersecting number between two samples represents the correlation of DNA methylation between them. (**B**) Methylated loci in genomic elements

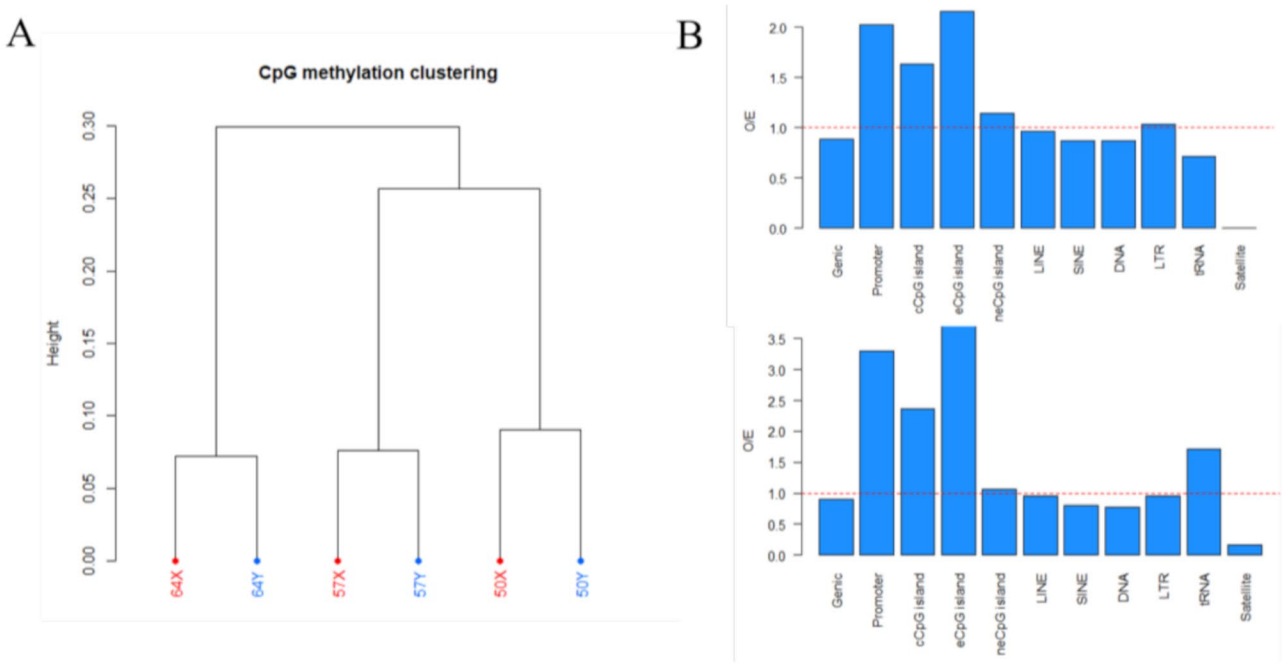

Fig. 2 Analysis of DMRs in bull X and Y Sperm. (**A**) Cluster analysis of DNA methylation data from 6 sperm samples. (**B**) Top: Distribution of hypermethylated regions in major functional elements of the genome. Bottom: Distribution of hypomethylated regions in major functional elements of the genome. The X-axis represented the main functional elements of the genome, and the Y-axis showed the ratio of observed to expected values. The observed/expected ratio was calculated as follows: the observed percentage (genomic elements with DMRs) was divided by the expected percentage (all genomic elements) of each genomic feature (e.g., genic, promoter) relative to the whole genome length

Given that previous studies have reported differences between X and Y sperm in terms of motility, cytoskeleton organization, energy metabolism, and associated protein expression [36–39], and that hypomethylated regions were more frequently enriched in tRNA compared to hypermethylated regions (Fig. 2B), we hypothesize that DNA methylation may regulate the expression of related genes, thereby contributing to these observed differences.

Identification of key DMGs associated with sperm epigenetics

Interestingly, we found that the hypermethylated genes *SPA17* and *CHCHD3* were previously reported as two of the 31 significantly differentially expressed membrane proteins in bovine X and Y sperm [40]. The *P* value for protein expression levels of *SPA17* and the *Q* value for its DNA methylation level were 0.0267 and 9.93×10^{-4}, respectively. For *CHCHD3*, the *P* value for protein expression levels and the *Q* value for its DNA methylation level were 0.0038 and 5.89×10^{-5}, respectively (Table 2).

In addition, we identified 28 genes related to spermatogenesis and spermatid development among the differentially methylated genes (DMGs), including 11 hypermethylated genes (*CELF1*, *OSBP2*, *ASZ1*, *PLEKHA1*, *GALNTL5*, *LIMK2*, *PATZ1*, *TYRO3*, *AK8*, *CCDC63*, and *FNDC3A*) and 17 hypomethylated genes (*ADAMTS2*, *DNMT3A*, *ERCC1*, *HERC2*, *HORMAD1*, *KIT*, *ACE*, *CEP57*, *CYP26B1*, *FSHR*, *GTSF1*, *MNS1*, *MEIG1*, *NANOS2*, *RFX2*, *SPATA20*, and *SPATA6*). Furthermore, we identified five fertilization-related genes, including two hypermethylated genes (*FNDC3A* and *FUT8*) and three hypomethylated genes (*PLCB1*, *SPADH1*, and *NECTIN2*).

Discussion

Comprehensive proteomic and transcriptomic studies have been conducted to elucidate the molecular differences between X and Y sperm. Although our group has identified variations in small non-coding RNAs, including miRNAs, Piwi-interacting RNAs, and tRNA-derived fragments, between X and Y sperm [17], the role of other epigenetic modifications, particularly DNA methylation, remains poorly understood. In this study, we employed WGBS to systematically compare the autosomal DNA methylation profiles of X and Y sperm from three fertile Holstein bulls. While global methylation profiles were conserved, localized analysis identified 2,041 DMGs enriched in energy metabolism and membrane voltage regulation—critical processes for sperm function. Intriguingly, hypermethylation of *SPA17* and *CHCHD3* in X sperm aligns with their reduced protein expression in X versus Y sperm, implicating epigenetic regulation. Key DMGs linked to spermatogenesis and fertilization further suggest DNA methylation may drive functional

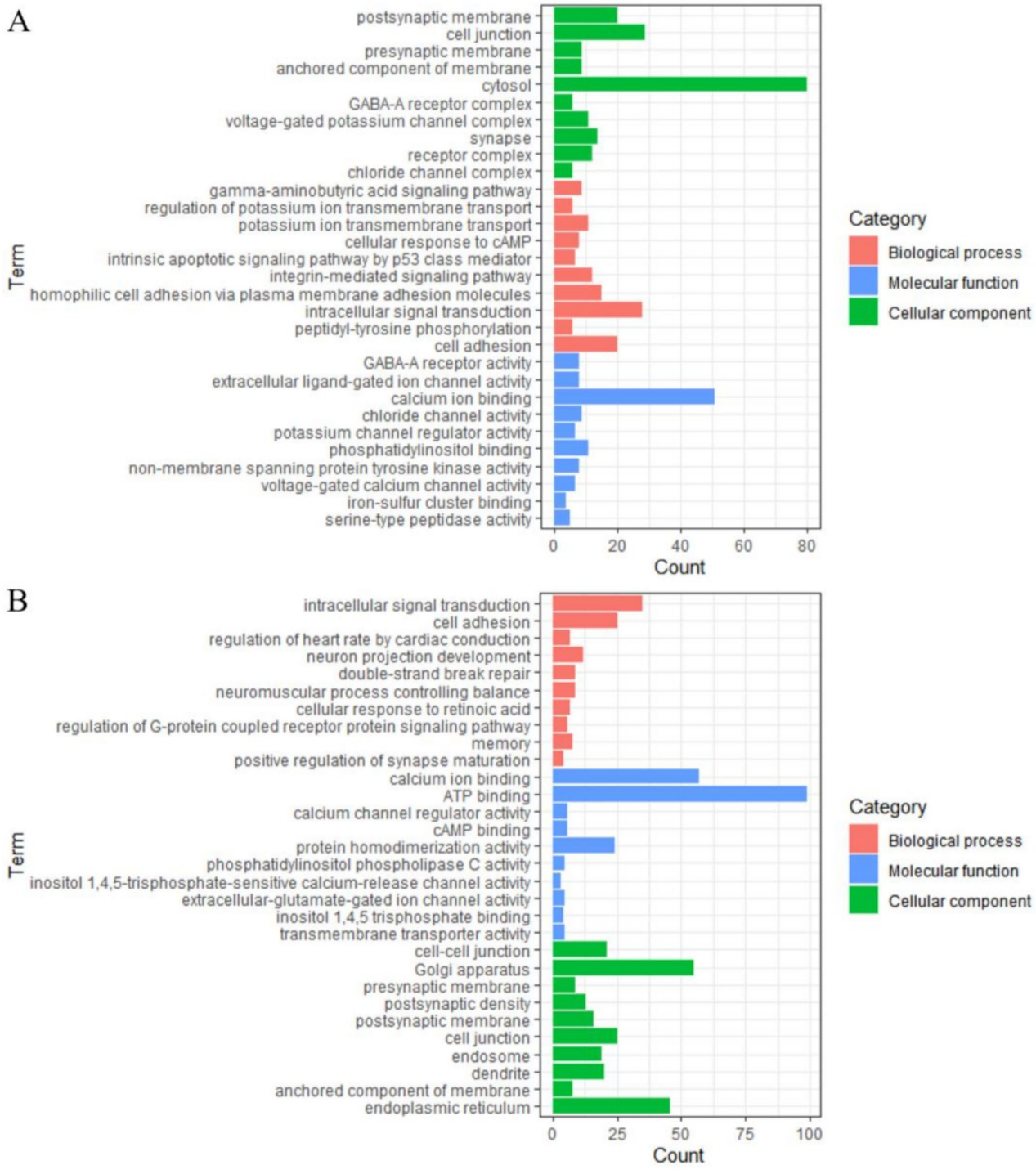

Fig. 3 GO enrichment analysis of genes overlapped with DMRs. (**A**) Enrichment analysis of hypermethylated DMGs. (**B**) Enrichment analysis of hypomethylated DMGs

divergence between X and Y sperm. These findings advance our understanding of sperm epigenetics and provide insights for potential sex control applications.

Compared to Y sperm, we identified 1,050 hypomethylated and 991 hypermethylated genes in X sperm. Among the hypomethylated genes, nine were imprinted genes, including *DIRAS3*, *CALCR*, *KCNQ1*, *ANO1*, *NTM*, *SLC38A4*, *RASGRF1*, *PEG3*, and *ISM1*. In contrast, five imprinted genes—*CTNNA3*, *SLC22A18*, *PPP1R9A*, *NTM*, and *HTR2A*—were found among the hypermethylated

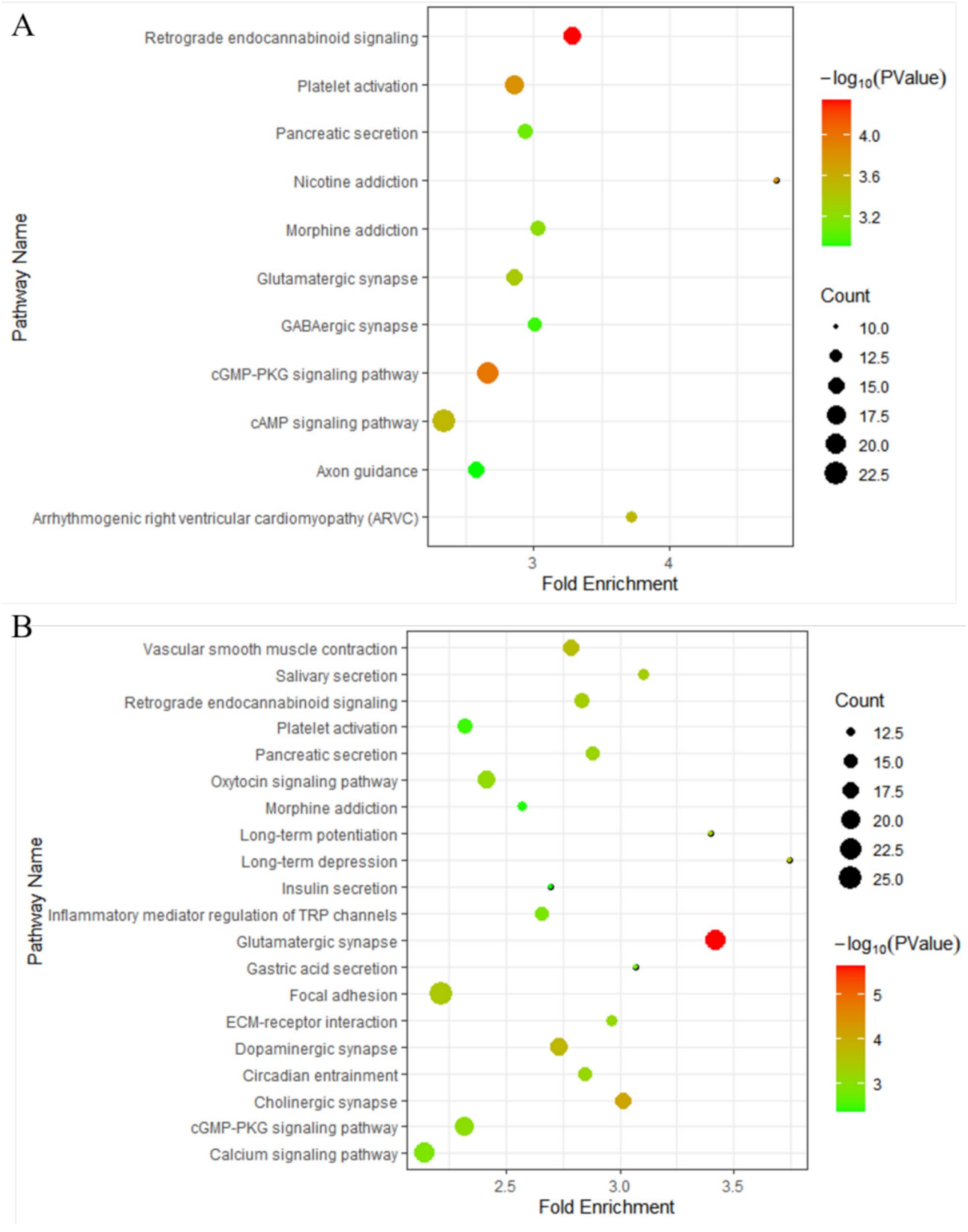

Fig. 4 KEGG enrichment analysis of genes overlapped with DMRs. (**A**) Enrichment analysis of hypermethylated DMGs. (**B**) Enrichment analysis of hypomethylated DMGs

Table 2 Differences in protein expression and DNA methylation level of *SPA17* and *CHCHD3* between X and Y sperm

Gene	Difference in protein level[1] (X vs. Y sperm)		Difference in DNA methylation level (X vs. Y sperm)	
	Ratio	*P* value	Q value	methylation difference
SPA17	0.71	0.0267	9.93×10^{-4}	31.11
CHCHD3	0.54	0.0038	5.89×10^{-5}	29.00

[1] The data were obtained from Shen et al. (2021) [40]

genes. Imprinted genes, which are expressed from a single parental allele and do not follow Mendelian inheritance, are widely conserved in mammals and play critical roles in embryonic development and placental function [41, 42]. The presence of imprinted genes among the DMGs further supports the robustness and reliability of our findings.

Sperm motility, capacitation, and fertilization involve dynamic changes in metabolism, cAMP signaling, calcium homeostasis, and pH, which are regulated by protein kinases and phosphatases [43, 44]. GO enrichment analysis identified numerous biological pathways associated with these factors, including calcium ion binding, potassium ion transmembrane transport, extracellular-glutamate-gated ion channel activity, cellular response to cAMP, and phosphatidylinositol phospholipase C activity, highlighting their involvement in the fundamental biological processes of sperm function. Interestingly, we observed that hypomethylated genes in X sperm were primarily enriched in ATP-binding pathway (97 genes), a finding that is consistent with previous proteomic studies comparing X and Y sperm [17, 40]. In our study, 14 genes within this pathway (*MAP3K9*, *MAP2K3*, *ACOT12*, *PGS1*, *eIF2AK2*, *PBK*, *RPS6KL1*, *PDXK*, *VPS4A*, *CAMKK2*, *RECQL4*, *CSK*, *LIMK1*, and *MAPK1*) harbored DMRs primarily in their promoter regions. The role of DNA methylation in regulating the expression of these genes, as well as the underlying mechanisms driving this biological process, warrants further investigation.

KEGG pathway analysis further revealed that differentially methylated genes were significantly enriched in pathways associated with hormone secretion and synaptic function. Notably, sex differences in DNA methylation were observed in pathways such as insulin secretion [23], morphine addiction [45], and long-term depression [46]. Given the established role of paternal epigenetic contributions in embryonic development, we speculate that the differential methylation of these genes may influence early developmental processes and contribute to the manifestation of sex differences.

Among the DMGs, the hypermethylated genes *SPA17* and *CHCHD3* were previously identified differentially expressed membrane proteins in bovine X and Y sperm [40]. The hypermethylation of these genes in X sperm corresponds with their lower protein expression levels, suggesting that DNA methylation may inhibit gene expression. Notably, both genes encode membrane proteins, which are essential for sperm function, including processes such as sperm motility, acrosome reaction, and fertilization. Specifically, SPA17 (sperm autoantigen protein 17) is a highly conserved mammalian protein primarily localized to the sperm plasma membrane and the fibrous sheath of the flagellum. Research has shown that *SPA17* is involved in sperm-egg interaction and may influence sperm motility and overall fertility [47, 48]. Interestingly, the observed hypermethylation of *SPA17* in X sperm could potentially impair its function, thereby contributing to the differences in sperm quality and motility between X and Y sperm. In addition, CHCHD3 (coiled-coil-helix-coiled-coil-helix domain-containing protein 3) is a key mitochondrial protein. This gene plays a critical role in regulating mitochondrial membrane potential and supporting ATP production [49, 50], both of which are essential for sperm viability, motility, and successful embryo development. The hypermethylation of *CHCHD3* in X sperm could potentially disrupt its function in mitochondrial activity, thereby further contributing to the observed differences in sperm characteristics between the X and Y sperm populations, as well as influencing the development of female embryos.

The findings of this study underscore the importance of epigenetic modifications, particularly DNA methylation, in regulating sperm function. The methylation of *SPA17* and *CHCHD3* suggests that these epigenetic changes may play a significant role in the regulation of sperm motility and fertilization potential. Given the crucial roles these proteins play in sperm function, further investigation into their methylation patterns could provide valuable insights into the mechanisms behind sex-specific differences in sperm characteristics.

Many studies have shown that DNA methylation is closely related to sperm quality [51], including sperm count, motility, and morphological abnormality rate, as well as spermatogenesis [11]. In our study, we identified 28 genes involved in spermatogenesis and spermatid development, such as *ADAMTS2*, *DNMT3A*, and *ERCC*, which have been implicated in key processes like germ cell differentiation, DNA repair, and hormone signaling [52–54]. Their differential methylation may influence spermatogenic efficiency and sperm maturation.

Additionally, sperm DNA methylation undergoes extensive reprogramming during zygote formation,

including global demethylation followed by partial region-specific re-methylation [55]. This dynamic process is essential for early embryonic development and genomic imprinting. Thus, investigating sperm DNA methylation provides valuable insights into fertilization success and embryonic development. In this study, we identified five fertilization-related genes (*FNDC3*, *FUT8*, *PLCB1*, *SPADH1*, and *NECTIN2*) that play important roles in acrosome reaction, zona pellucida binding, and oocyte penetration [56, 57]. The differential methylation of these genes may affect fertilization efficiency and early embryonic viability, highlighting the need for further investigation into their regulatory mechanisms.

While this study provides novel insights into autosomal methylation differences between bull X and Y sperm, several limitations should be acknowledged. First, although flow cytometry is widely used in both research and commercial applications with a reported separation accuracy exceeding 90%, the incomplete purity of sorted X and Y sperm may introduce some degree of bias into downstream differential methylation analyses. To minimize this, we focused exclusively on DMRs consistently identified across all three bulls, thereby reducing potential contamination effects. Second, we did not assess methylation profiles before and after sorting, which would be valuable for determining the extent to which the sorting process influences DNA methylation. Third, while genes such as *SPA17* and *CHCHD3* exhibited statistically significant methylation differences, our study did not directly evaluate their potential as definitive biomarkers for sperm sexing. It is important to emphasize that this study was not intended to develop a methylation-based sexing methodology but rather to identify autosomal epigenetic signatures that may be associated with functional differences between X and Y sperm. Fourth, the limited sample size ($n = 3$ bulls) constrains the generalizability of our findings, highlighting the need for validation in larger cohorts with diverse genetic backgrounds.

To advance this field, future research could focus on several key aspects. First, systematically integrating genomic, transcriptomic, proteomic, and epigenomic data will facilitate a deeper understanding of the functional significance of identified DMRs in X and Y sperm and aid in the discovery of novel biomarkers for sperm sorting. Second, validating candidate DMRs across genetically diverse populations and incorporating machine learning algorithms to establish standardized methylation thresholds resistant to individual variability will be critical for the practical application of biomarkers in reproductive management. Although the application of methylation biomarkers in livestock breeding remains challenging due to the dynamic nature of epigenetic modifications and the high cost of large-scale validation, advancements in single-cell epigenomics and microfluidic technologies provide promising avenues for developing methylation-based sorting approaches that may surpass traditional physical separation techniques.

Conclusion

The differences in DNA methylation between X and Y sperm in mammals remain to be fully elucidated. In this study, we systematically compared the DNA methylation profiles of bull X and Y sperm, identifying differentially methylated genes and regions. These findings provide foundational insights into the epigenetic distinctions between X and Y sperm, contributing to the understanding of sperm sex differentiation and the broader field of epigenetic regulation in sperm.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11402-6 .

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
S.Z. and Y.Z. designed and directed all the research. A.S., F.D., Q.X., and M.C. conducted the data processing and experimental analysis. R.D., W.S., X.L. X.B., T.Z. and H.C. collected and processed the semen samples. Y.Z., A.S. and F.D. drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

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Data availability
The raw sequencing data in this study were submitted to Sequence Read Archive (SRA) of National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, Bioproject number: PRJNA797921).

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Huazhong Agricultural University (permit number: HZAUSW-2017-012).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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