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DNA methylation differences between male and female gonads of the oyster reveal the role of epigenetics in sex determination

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ABSTRACT

DNA methylation involved in sex determination mechanism by regulating gene expression related to sex determination networks are common in vertebrates. However, the mechanism linking epigenetics in invertebrates and sex determination has remained elusive. Here, methylome of the male and female gonads in the oyster *Crassostrea gigas* were conducted to explore the role of epigenetics in invertebrate sex determination. Comparative analysis of gonadal DNA methylation of females and males revealed that male gonads displayed a higher level of DNA methylation and a greater number of hypermethylated genes. Luxury genes presented hypomethylation, while housekeeping genes got hypermethylation. Genes in the conserved signaling pathways, rather than the key master genes in the sex determination pathway, were the major targets of substantial DNA methylation modification. The negative correlation of expression and promoter methylation in the diacylglycerol kinase delta gene (*Dgkd*) - a ubiquitously expressed gene - indicated DNA methylation may fine turn the expression of *Dgkd* and be involved in the process of sex determination. *Dgkd* can be used as an epigenetic marker to distinguish male *C. gigas* based on the different methylation regions in the promoter region. The results suggest that DNA methylation mechanisms played potential functional impacts in the sex determination in oysters, which is helpful to deepen the understanding of sex determination in invertebrate.

1. Introduction

Sex determination refers to the mechanisms that direct sexual/gonadal differentiation, and sex differentiation is the development of ovaries or testes from the undifferentiation or biopotential gonad (Hayes, 1998). The mechanisms of the sex determination are divided into genetic sex determination (GSD) and environmental sex determination (ESD) (Hayes, 1998). Sex determination of mammals, birds and fish with sex chromosomes is mainly affected by GSD commonly showing male heterogamety (XY) or female heterogamety (ZW) mechanisms (Koopman, 2001). The expression or non-expression of sex determining genes is decisive instrumental for the direction of gonad development. However, in some invertebrates with or without sex chromosomes, sex determination is the result of a combination of genetics and environment. Epigenetic mechanisms, including DNA methylation, histone acetylation, chromosome remodeling and RNA interference, provide organisms with the ability to integrate genomic and environmental information to modify the activity of the genes for generating a particular phenotype (Cheng et al., 2019; Feng et al., 2010; Piferrer, 2013). In vertebrates, epigenetic regulation of gene expression during gonadal differentiation could provide evidence for sex reversal (Manolakou et al., 2006). For instance, high temperature can increase the DNA methylation levels of the promoter regions of *Cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a) and *Dmrt*, leading to suppressed gene expression and male development (Navarro-Martín et al., 2011; Shao et al., 2014). In addition, other genes in the sex

Abbreviations: BSP, Bisulfite-PCR; DEGs, differentially expressed genes; DGKD, diacylglycerol kinase delta; DMGs, differentially methylated genes; DMRs, differentially methylated regions; DMRT, doublesex and mab-3 related transcription factor; ESD, environmental sex determination; FG, female gonads; FEM 1C, feminization-1c; FOXL2, forkhead box l2; FPKM, Fragments per kilobase per million reads; GSD, genetic sex determination; INSR, insulin-like peptide receptor; MG, male gonads; MSP, methylation-specific PCR; *Sox9*, SRY-box transcription factor 9; *SoxH*, SRY-box transcription factor H; STPG2, sperm tail PG-rich repeat-containing protein 2; VG, vitellogenin; *Wnt4*, Wnt Family Member 4.

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determination pathway are also directly regulated by DNA methylation (Shao et al., 2014). However, information on the epigenetic mechanisms in sex determination is still lacking in invertebrates.

In the past decades, neither karyotype analysis nor genomics has found sex chromosomes in bivalves (Peng et al., 2020; Qi et al., 2021; Zhang et al., 2012). Interestingly, sex related genes (*Foxl2*, *Wnt4*, *β-Catenin*, *Dmrt*) found in vertebrates were also identified in bivalves, and only a few genes (*Foxl2* and *Dmrt*) show sexual dimorphism at transcriptional level (Li et al., 2018; Zhang et al., 2014). Indeed, whether sex is controlled by GSD or ESD in bivalves remains unclear. The hypothesis that the sex is determined by two genotypes and three genotypes has been proposed in oysters (Teaniniuraitemoana et al., 2014; Guo et al., 1998; Hedrick and Hedgecock, 2010).

The Pacific oyster *Crassostrea gigas*, which is a continuously hermaphrodite species, shows successive sex reversal during their life history (Broquard et al., 2020). Most oysters initially show males maturing first and then reversing to females, or vice versa (Amemiya, 1929). In addition to genetic factors, environmental factors have a great influence on the sex differentiation of bivalves. For example, warmth and food availability conditions favor the development of females (Zhang et al., 2014). Therefore, sex determination might be also regulated by epigenetics in bivalves. The individual with sex reversal is unchanged in DNA sequence, but the phenotype is altered, suggesting that epigenetics is also involved in the process of sex reversal (Rivière, 2014). DNA methylation is a pivotal epigenetic modification associated with phenotypic plasticity that allows oysters to adapt to unpredictable conditions (Rivière, 2014). Unlike other invertebrates, oysters show high levels of DNA methylation involved in gene regulation (Olson and Roberts, 2014; Wang et al., 2014). These indicate that oysters exhibit specific characteristics in DNA methylation, suggesting more phenotypic plasticity including gonadal plasticity (Rivière, 2014; Roberts and Gavery, 2012). Epigenetic mechanisms of sex determination have been studied in many vertebrates (Navarro-Martín et al., 2011; Ortega-Recalde et al., 2019; Shao et al., 2014), but relatively few in invertebrates. Oysters provide a good biological model based on complete genomic information and biological characters for investigating the role of epigenetics in sex determination in bivalve. The purpose of this work is to explore the mechanism of epigenetics in sex determination of *C. gigas*. Therefore, we compared differences of DNA methylation in female and male gonads and displayed the methylation status of the sex related genes.

2. Materials and methods

2.1. Animals

In June, 14-month-old oysters were collected from a local oyster farm in Yantai, Shandong Province, China. Sex and gonadal development stage were identified by histological observation. The mature gonads of three females and three males were immediately frozen in liquid nitrogen, and stored at −80 °C before DNA methylation and RNA-seq analysis. We also sampled 30 females and 30 males in order to conduct methylation-specific PCR (MSP) and Bisulfite-PCR (BSP) analysis.

2.2. Sequencing and data analysis of DNA methylation

Genomic DNA were extracted from three female gonads (FG) and three male gonads (MG) using a DNA extraction kit (Tiangen, China) according to the manufacturer's instructions. Then 5 μg genomic DNA was utilized to perform bisulfite conversion and BS-seq. Lambda DNA was added to the genome to detect the bisulfite conversion efficiency. The raw data were submitted to the NCBI Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/subs/sra/) and the accession number were SRR13305464, SRR13305463, SRR13305454, SRR13305447, SRR13305462 and SRR13305461.

BSMAP (version: 2.90, default parameters) software was applied to map the BS-seq reads to the reference genome as described in the study (Xi and Li, 2009), allowing no more than 4 mismatches for 90 bp reads. The methylation level of a given region was calculated by dividing the sum of the methylation levels of individual CGs in the region by the total number of CGs covered in the region. Methylation levels were calculated by the R scripts.

2.3. Differentially methylated regions (DMRs) analysis for comparing between FG and MG

DMRs were identified using the DSS software and the minimum read coverage to call the methylation status for a base was set to 4 according to the previous study (Wu et al., 2015). The average DNA methylation rate in each window was calculated using the sliding-window approach with 200 bp window slide at 50 bp intervals. Only the numbers of CG in each window ≥ 5, absolute value of the difference in methylation ratio ≥ 0.25, and $q < 0.05$ were considered as DMRs. The a methylkit R package (Akalin et al., 2012) was utilized to DMRs annotation.

2.4. Enrichment analysis of DMR-related genes

To analyze functional enrichment of genes affected by DMRs, Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted for DMR-related genes. GOseq R package (Young et al., 2010) was implemented to GO enrichment analysis of DMR-related genes. GO terms with corrected $P < 0.05$ were considered significantly enriched by DMR-related genes. KEGG pathway enrichment analysis was conducted for DMR-related genes by means of KOBAS software (Mao et al., 2005) and $q < 0.05$ were considered significantly enriched.

2.5. RNA-seq and data analysis

Total RNAs were isolated from the oyster gonads using the TRIzol reagent ((Invitrogen) according to the manufacturer's protocols. The libraries were constructed as previously described (Yue et al., 2018) and were then sequenced on an Illumina HiSeq™ 4000 platform and paired-end reads with an average length of 150 bp were generated. TopHat2 (v 2.0.3.12) package was employed to map transcriptome data to the oyster genome with default parameters. Gene expression levels were normalized using FPKM (Fragments per kilobase per million reads). The raw data were submitted to the NCBI Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/subs/sra/) and the accession number were SRR13374889, SRR13374888, SRR13374879, SRR13374872, SRR13374887 and SRR13374886.

2.6. Correlation of DNA methylation and gene expression between groups

To analyze whether differentially expressed genes (DEGs) influenced by DNA methylation between groups, DEGs were divided into four classes based on their expression levels, including a specific-down group (FPKM < 1 in MG and ≥ 1 in FG), and a specific-up group (FPKM < 1 in FG and ≥ 1 in MG), an other-down group (genes nonspecific down-regulated expressed in MG), an other-up group (genes nonspecific up-regulated expressed in MG).

To determine whether DNA methylation level in differentially methylated regions (DMRs) influences gene expression between groups, genes were classified according to genomic location, including the ±2 kb flanking regions and genebody regions.

2.7. MSP detection for the relationship between methylation of sex-related genes and Dgkd and genders

The genomic DNA extraction kit (Tiangen, China) was used to extract genomic DNA from 30 females and 30 males according to the

instructions of the kit. The concentration and purity of DNA were determined by UV spectrophotometry and stored at − 80 °C. DNA bisulfite transformation kit (Tiangen, China) was used to treat DNA with sodium sulfite. The purified DNA could be used for subsequent MSP reaction. Methylated-specific primers and unmethylated-specific primers were designed for DMR regions (Table 1). 2 × taq master mix enzyme (Vazyme, China) was applied for PCR amplification. The reaction procedure was pre denaturation at 95 °C for 10 min; denatured at 95 °C for 15 s, 58 °C for 20 s, annealed at 72 °C for 30 s, 35 cycles; finally, it was extended at 72 °C for 10 min. The products were analyzed by agarose gel electrophoresis, and the number of specific methylation bands between male and female was counted and the significance was analyzed by chi-square test. $P < 0.05$ was statistically significant.

2.8. DNA methylation of Dmrt validation in females and males by BSP

The BSP templates were the DNA of the above transformation including three females and three males. PCR amplification was performed using the LA taq enzyme (TaKaRa) and the Me*Dmrt* primers (Table 1), then the target fragment was ligated to the vector pMD19-t (TaKaRa). Randomly pick ten clones for each sample for sequencing. Software BiQ_Analyzer was used to analyze the sequencing results and graph.

3. Results

3.1. DNA methylation difference between FG and MG

The conversion efficiency of all the samples treated by bisulfite was more than 99%, indicating the reliability of the experiment. Whole-genome bisulfite sequencing was applied to genomic DNA of oysters extracted from gonads. In total, 333 million reads were generated for male gonad tissue and 341 million reads were generated for female gonad. Of the reads generated in this study, 199 (59%) million reads from the male gonad and 188 (57%) million reads from the female gonad could be aligned to the reference genome of oyster (Table S1). To identify divergence in DNA methylation, we performed comparisons to identify the differentially methylated regions between male and female gonads. Apparently, the methylation levels of most CG sites in FG were lower than those of MG (Fig. 1 A). In total, 12,236 DMRs at CG sites with 1,018 DMRs in upstream, 10,441 DMRs in genebody and 777 DMRs in downstream were discovered, and 10,534 (86%) and 1,702 (14%) DMRs were hypermethylated in MG and FG, respectively (Fig. 1B and Table S2). It is clear that the number of hypermethylated DMRs in male gonads was higher than that of hypermethylated DMRs in female gonads, especially in gene body region (Fig. 1 B). As CG methylation was the dominant methylation context in the samples and only 1 CHG site DMR and 15 CHH site DMRs were discovered in the compared groups, we focused on the investigation of CG DMRs.

The regulatory network of genes from differentially methylated genes (DMGs) was constructed to reveal the GOs and signaling pathways regulated by differentially methylated genes, and to understand the physiological function and biological process of DMGs in gonadal activity. GO enrichment analysis showed that the most significant functional terms were kinase activity, phosphotransferase activity, alcohol group as acceptor, protein kinase activity and transferase activity, transferring phosphorus-containing groups (Fig. 1C). KEGG enrichment analysis of DMGs were also conducted. As a whole, the DMGs were significantly enriched in 32 KEGG pathways, with $q < 0.05$. The KEGG pathways with the most genes related to DMGs included MAPK signaling pathway (ko04010), with 70 genes, followed by Ras signaling pathway (ko04014), with 57 genes and Regulation of actin cytoskeleton (ko04810), with 51 genes. In addition, 11 signaling pathways related to gonadal development, including Phospholipase D signaling pathway (ko04072), Hedgehog signaling pathway (ko04340), Fatty acid biosynthesis (ko00061) (Fig. 1 D, Table S3).

3.2. Association between DNA methylation and gene expression

A total of 213 and 162 million reads were generated from male and female gonads, respectively, with the unique mapping ratio of 72% (Table S4). To better understand the correlation between the variation of DNA methylation and gene expression in groups, we divided the genes into specific-up regulated genes, specific-down regulated genes, other-up genes and other-down genes (see materials and methods) according to their differential expression levels (Table S5). As expected, the methylation levels of non-specifically expressed genes throughout much of gene region presented high methylation levels, especially in gene body region (Fig. 2 A, B). By contrast, the specifically expressed genes presented low methylation levels. That means ubiquitously expressed critical genes are highly methylated and the tissue specific expressed genes were low methylated, in line with previous study (Roberts and Gavery, 2012). For further explore the potential consequences of DNA methylation on gene expression, we detected the overlapping genes of DMRs and DEGs. Combined with the transcriptome data, 18% (618 out of 3496) of genes that overlapping with DEGs and DMRs were discovered in FG vs MG (Fig. 2C, Table S6). Non-specific regulaion genes accounted for 72% of genes, whereas 10% and 12% of specifically up-regulated genes harbored DMRs in FG and MG, respectively (Fig. 2D). These data proved that DNA methylation regulated minor number of genes, of which the majority were ubiquitously expressed.

Among the above 618 genes, 517 genes were significantly enriched in 30 KEGG pathways and more than one-third have to do with signal transduction, including Phospholipase D signaling pathway, ErbB signaling pathway, MAPK signaling pathway - fly and so on (Fig. 2E, Table S7). The gene encoding insulin-like peptide receptor (INSR) was enriched in 9 of the 30 pathways and displayed hypomethylated and high expressed in females (Table S6). The diacylglycerol kinase delta gene (*Dgkd*), which was enriched in Phospholipase D signaling pathway and Phosphatidylinositol signaling system (Table S6), was hypomethylated and up expressed in females, suggesting that demethylation activated the expression of gene related to metabolism.

Table 1
MSP and BSP primers used in this study.

Genes	Sense primer	Antisense primer	Size (bp)	Anneal temperature (°C)
Vgf-M	TCGAAATTAAAATAGTGTTTGTACGT	AAAACAACCGATAATTAAACGAA	173	60
Vgf-U	ATTGAAATTAAAATAGTGTTTGTATGT	AAAACAACCAATAATTAAACAAA	172	53
Insr-M	TTGTAAATATTTAAGGATAGGTCGA	AAATAATCGATTTCATACACTCGTA	219	57
Insr-U	TTGTAAATATTTAAGGATAGGTTGA	AAATAATCAATTTCATACACTCATA	219	57
Stpg-M	TAGAAATTTCGTAAAAGAAAAATTACGT	TAACGAAAAACATAACTAAAACGAC	115	57
Stpg-M	ATAGAAATTTTGTAAAAGAAAAATTATGT	TAACAAAAAACATAACTAAAACAAC	116	57
Dmrt-M	ATGTCGGGAAAATGTATTTTTTTC	AATTTCGTACAACTTATCCAACGTA	110	57
Dmrt-U	TGTTGGGAAAATGTATTTTTTTTG	AATTTCATACAACTTATCCAACATA	109	57
Dgkd-M	TTTGTATTATTTAAGTTTTAATACGA	AATAACATCTCTATTATTTATCACGAT	212	57
Dgkd-U	AAATTTGTATTATTTAAGTTTTAATATGA	AATAACATCTCTATTATTTATCACAAT	215	57
Me*Dmrt*	AAGTGATGTTTGGTAATATTATTA	ACTATCCCTAACTATTTTCTCTTTTCTA	207	57

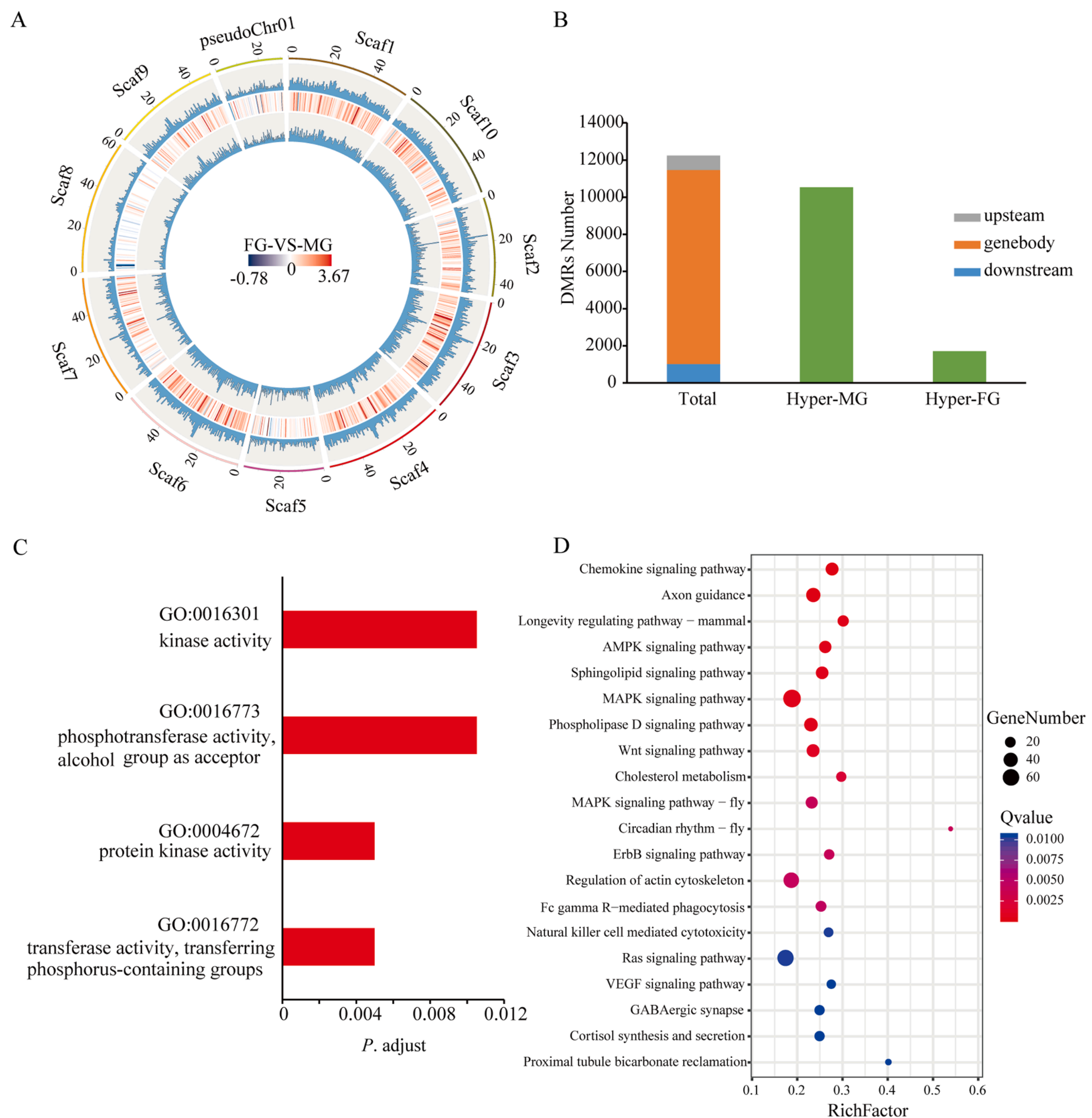

Fig. 1. Comparative methylome analysis of male and female gonads. **A** Circos plot of genome scaffolds. The three circles from outer to inner represented the methylation levels of MG, the differences between FG and MG, and FG, respectively. The level of methylation is denoted by the darkness of the color, and the level of the difference is represented by the heatmap. **B** The differentially methylated regions (DMRs) number in different gene regions, hypermethylated in MG (Hyper-MG) and hypermethylated in FG (Hyper-FG). **C** Gene ontology (GO) of differentially methylated genes (DMGs) in functional terms. **D** Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of DMGs by hierarchical clustering. Rich factor means that the ratio of the number of the DMGs in the specific subcluster and the number of DMGs annotated in this pathway.

3.3. DNA methylation of sex related gene in oysters

By comparing the diversity between the methylation groups of male and female gonads, it is expected to find the sex-determining genes regulated by methylation. We investigated the methylation states of 9 genes related to sex determination and gonadal development in oysters, as documented in previous studies. Overall, three out of the nine sex-related genes in oysters displayed significant differential methylation patterns between FG and MG. Surprisingly, the sex-specific expressed genes (*Foxl2*, *Dmrt*, *Sry*, *Nanos1*, *Fem1c* and *Vg*) showed hypomethylation throughout much of the gene except *Fem1c* (Fig. 3 A-D, F and H). However, the non-specifically genes (*Insr*, *Sox9* and *Stpg2*) involved in gonadal development presented hypermethylation in the entire gene region, with the exception of *Sox9* (Fig. 3 E, G and I). This further demonstrated that universally expressed genes tend to be hypermethylated, while specifically expressed genes were demethylated. The two genes encoding INSR and vitellogenin (VG) essential for the energy storage of late gonads in females displayed demethylation in gene body and upstream 2 kb region, respectively. The sperm tail PG-rich repeat-containing protein 2 gene (*Stpg2*) was related to spermatogenesis, but it showed high expression and hypermethylation in males.

MSP primers of *Insr*, *Stpg2* and *Vg* were designed to analyze the association between methylation sites and gender. MSP and BSP primers of *Dmrt* were also designed as the methylation sites existed in the upstream of the second sample. Females and males DNA treated with sulfite were used as templates for PCR amplification. The results showed that very

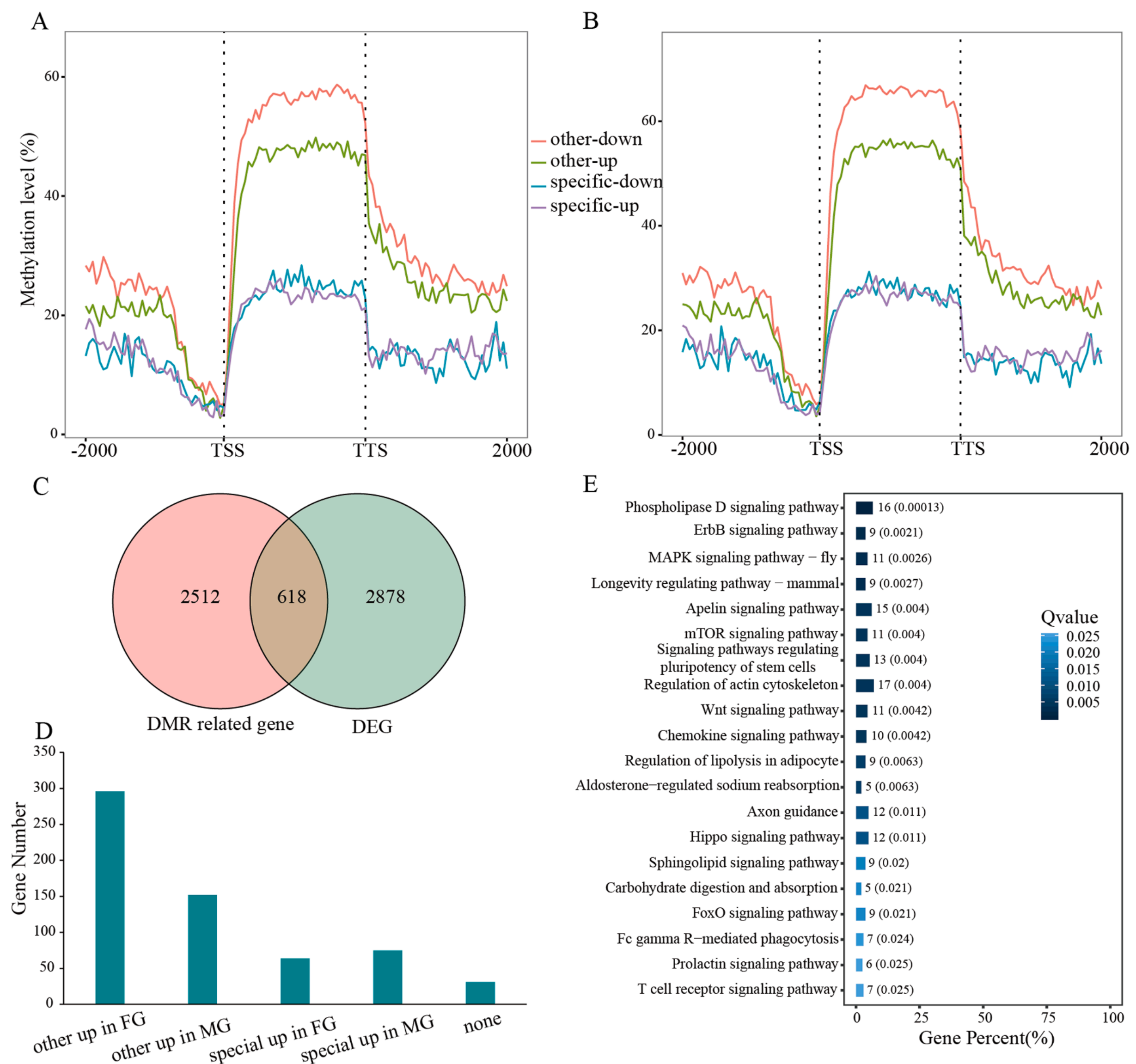

Fig. 2. Association between DNA methylation and gene expression. **A, B** Distribution map of genes with different differential expression levels and DNA methylation levels in female and male, respectively. The abscissa represents the position from upstream 2 kb (-2000) to downstream 2 kb (2000), TSS and TTS represent the transcription start site and the transcription stop site respectively, and the ordinate represents the average methylation level. **C** The Venn diagram shows the number of DMR related genes, the number of DEGs and the number of genes they intersect. **D** The number of DEGs harbored DMRs in different differential expression levels. **E** KEGG annotation of DEGs harbored DMRs by hierarchical clustering.

little methylation occurred in *Dmrt* (Fig. 3 K and L), which is consistent with the BS-seq results, indicating the methylation sequencing results are reliable. The MSP of *Dmrt* could not amplify methylated bands, but could amplify unmethylated bands in female and male (Fig. 3M) and almost no methylation sites were present in upstream. In contrast, *Insr* and *Stpg2* were methylated in females and males, and *Vg* was unmethylated in females, but methylated in males. The methylation status of 30 females and 30 males were measured and the statistical results were as follows. No methylation of *Dmrt* was detected in all the samples. On the contrary, *Insr* and *Stpg2* undetected methylation in the 60 samples. Regarding *Vg*, methylation was detected in 8 female samples and 18 male samples, and chi-square test showed that the methylation sites of *Vg* were significantly different between males and females ($P = 0.018$). But the unmethylated states persisted, meaning that the methylation levels were not 100% in samples tested. These results raise the possibility that sex difference were related to the regulation of none-sex-determining genes by DNA methylation.

3.4. DNA methylation of Dgkd was sensitive to gender and repress gene expression

Here, we observed that *Dgkd* harbored a DMRs on the promoter region that was hypomethylated compared to males, which might correspond to its approximately 2.5-fold up-regulation expression in females (Fig. 4 A and B). To further inspect the correlation between *Dgkd* methylation and sex, we examined gene methylation in 30 females and 30 males using MSP method. No methylation bands were detected in females, but methylation bands were detected in males, indicating that no methylation occurred in females and methylation occurred in males (Fig. 4C). Subsequent testing of 30 females and 30 males showed that no methylation bands were detected in 30 females, while methylation occurred in 21 males. It thus appears that the expression of *Dgkd* might be regulated by DNA methylation and that *Dgkd* could serve as a potential sex marker for sex discrimination in oysters.

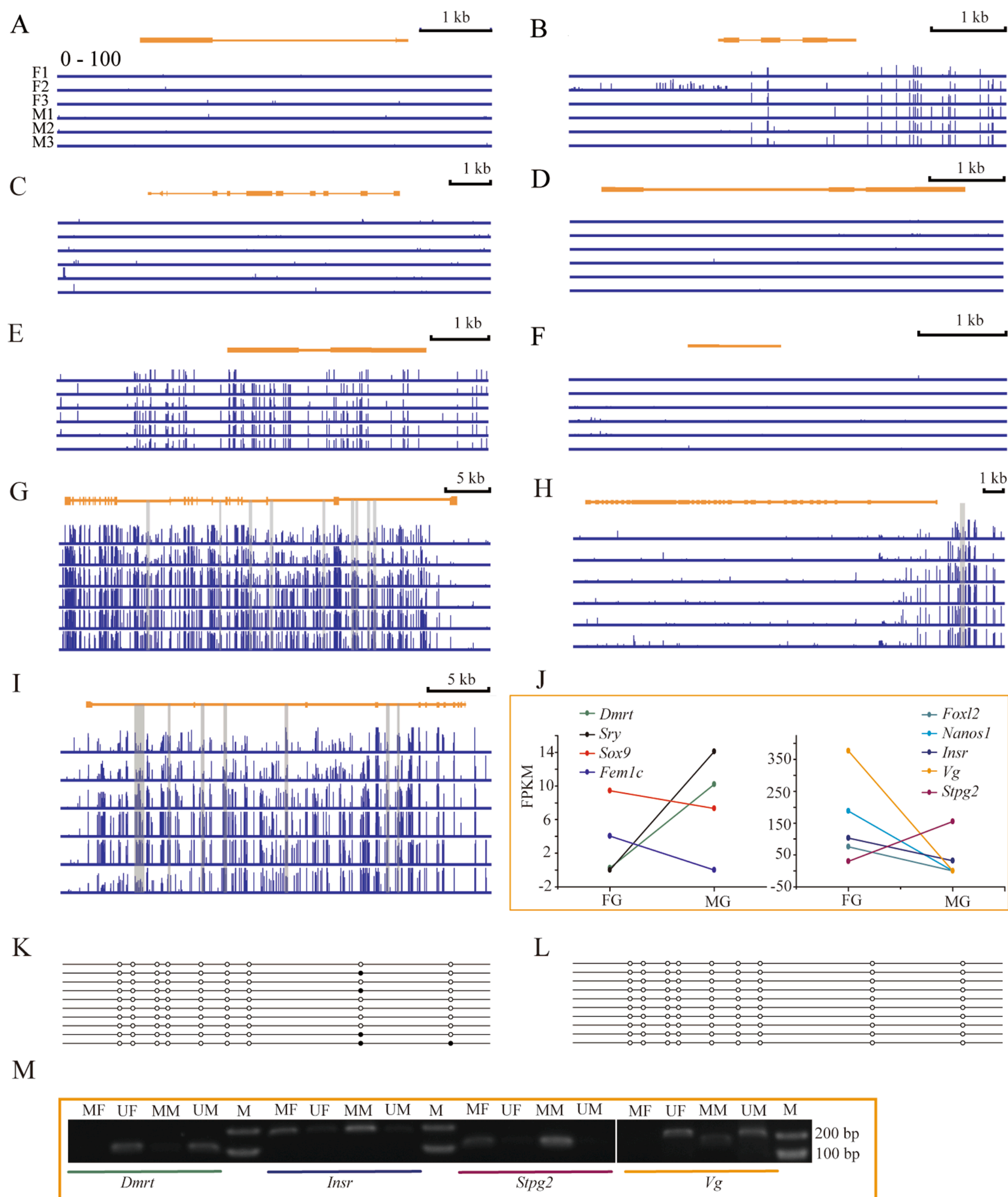

Fig. 3. DNA methylation profiles of sex-related genes. **A-I** The methylation profiles of *Foxl2*, *Dmrt*, *Sry*, *Sox9*, *Fem1c*, *Nanos1*, *Insr*, *Vg* and *Stpg2*. **J** The FPKM of nine genes related to sex in female gonads (FG) and male gonads (MG). **K, L** Validation of *Dmrt* methylation in females and males, respectively. **M** MSP analysis of *Dmrt*, *Insr*, *Stpg2* and *Vg*. Primer sets used for amplification are designated as methylated in females (MF) or males (MM) and unmethylated in females (UF) and males (UM). M represents marker. The gray box indicates the DMRs area in **A-I**.

4. Discussion

Our study suggested that DNA methylation is indirectly involved in the regulation of sex differentiation pathway in oysters. According to genome-wide methylation analysis, significant methylation differences were observed between gonads of male and female individuals, with the master genes in the sex-determination pathway displaying hypomethylation. Analysis of the methylome indicated that DNA methylation was mainly related to housekeeping genes in oysters. Collectively, our data demonstrated that DNA methylation was involved in the sex differentiation pathway of oysters through the regulation of housekeeping genes.

Comparative methylomes for studying epigenetic effects on sex differentiation have been widely reported (Chen et al., 2017; Shao et al., 2014; Zhou et al., 2019), but limited studies in invertebrates. In this study, higher methylation levels were found in male gonads than female

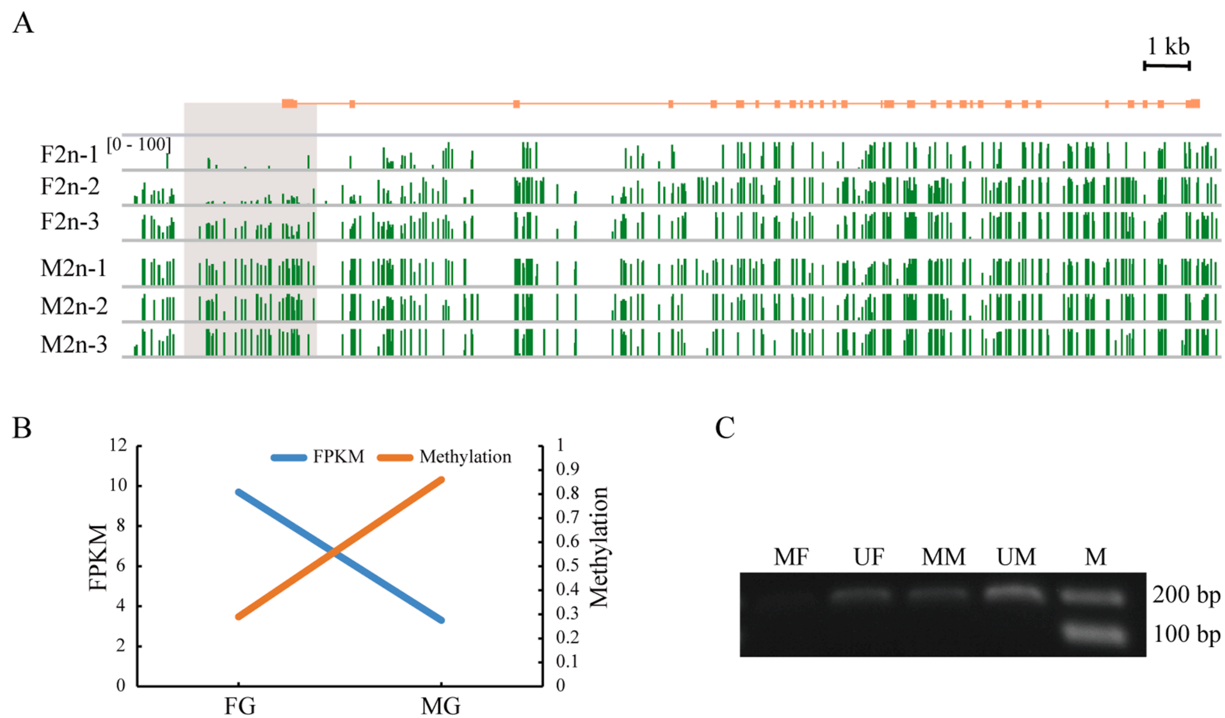

Fig. 4. Expression and methylation difference of *Dgkd* between female and male. **A** DNA methylation profiles of *Dgkd* in gonadal samples. **B** The expression and DNA methylation levels of *Dgkd*. **C** MSP analysis of *Dgkd*. Primer sets used for amplification are designated as methylated in females (MF) or males (MM) and unmethylated in females (UF) and males (UM). M represents marker. The gray box indicates the DMRs area in **A**.

gonads by comparative methylomes, in line with previous studies of higher methylation in testis than in ovaries using fluorescence-labeled methylation-sensitive amplified polymorphism technique (Zhang et al., 2018). The DMRs between FG and MG mostly occur in the gene body region, indicating that DNA methylation modified gene body region were involved in sex differentiation. KEGG enrichment analysis showed that DMGs were enriched in metabolic and signaling pathways in oysters, whereas sex-specific DMGs were enriched in pathways related to gonadal development and sex determination in vertebrates (An et al., 2018; Zhong et al., 2013). In addition, the recent work has found that sex differences in DNA methylation are not the prerogative of gonadal tissue, and that differences in global DNA methylation levels in muscle were also gender-related (Li et al., unpublished data). Similarly, differential DNA methylation in brain can affect the reproductive and behavioral changes in queens and workers honeybees (Lyko et al., 2010). This suggested that there are inherent differences in the methylome of male and female oysters. Environmentally induced sex reversal may be an event of altered methylome at the whole-body level of the individual, rather than just an increase or decrease in methylation levels in gonadal tissues. Inherent differences may also be present at the transcriptome level, which is found between male and female rainbow trout (Hale et al., 2011), but may be overlooked between female and male oysters. Thus, DNA methylation is involved in the sex determination of the Pacific oyster.

The relationship between DNA methylation and gene expression can clearly indicate the biological significance of gene expression types and DNA methylation levels. Indeed, in the study, widely expressed genes got hypermethylated, whereas the specifically expressed genes were hypomethylated, which is consistent with previous reports that CpG methylation preferentially targets housekeeping genes (Gavery and Roberts, 2010; Roberts and Gavery, 2012). However, not all specifically expressed genes were hypomethylated, unravelling a precise regulation of DNA methylation. Such regulation was marked in female and male gonads, implying a participation of methylome in regulating these genes. In addition, DNA methylation was associated with the expression of ~18% DEGs. The ratio of DEGs with DMRs in oysters differed from the ratio of DEGs found in mammals, where 60–80% DEGs, not just the ubiquitously expressed genes, harbored DMRs (Shao et al., 2014; Zhang et al., 2019). Housekeeping genes tend to be evolutionarily conserved genes, and methylation of these genes contributes to increased phenotypic plasticity in highly volatile environments (Roberts and Gavery, 2012). KEGG enrichment analysis for DEGs with DMRs revealed that these genes were significantly enriched in evolutionarily conserved signaling pathways, including Phospholipase D signaling pathway, ErbB signaling pathway, MAPK signaling pathway - fly, etc. Among gonadal DEGs, hypermethylated DEGs were housekeeping genes, and most of the DEGs with DMRs were also housekeeping genes, implying that sex differentiation is related to the regulation of housekeeping gene expression by DNA methylation.

Sexual differentiation is modulated by the network of genes that have synchronously activate downstream cascades in vertebrates (Angelopoulou et al., 2012). The master genes in the network are highly conserved in evolution and have plastic expression across species (Angelopoulou et al., 2012). Functionally conserved genetic components involved in gonadal differentiation in GSD animals also play a role in the expression of ESD through epigenetic regulation (Shao et al., 2014). It has been proposed that *Foxl2* and *Dmrt*, as key master genes, are instrumental in the network of sex determination in oysters (Zhang et al., 2014). However, the epigenetic regulatory mechanisms of sex determination in oysters differ from those expected in vertebrates. The conserved specifically expressed genes including *Foxl2*, *Sry*, *Nanos1*, *Sox9* and *Dmrt* were almost demethylated in the sex determination pathway. As a sex-determining gene, *Dmrt* exhibits high methylation levels in vertebrates, and methylation at the promoter locations differs between males and females, which determines the expression level of *Dmrt* and further determines the direction of sex development (Shao et al., 2014). In addition, the other sex-determination-related genes (*Cyp19a1a*, *Vasa*) display significantly different methylation patterns between testes and ovaries (Shao et al., 2014). For these reasons, we ruled out the function of transient methylation in the regulation of sex-

related genes. Whereas other genes related to gonadal development were methylated, *Insr*, *Vg* and *Stpg2* displayed significant differences in gonadal expression and methylation levels in oysters. Given that the oyster is a hermaphrodite species and the complexity of its environment, limited methylation may contribute to increased phenotypic plasticity (Roberts and Gavery, 2012), and strategies of hypomethylation for sex-related genes make sex maintenance less susceptible to environmental fluctuations. During sex reversals, methylation of genes required for normal biological functions, such as energy metabolism, may respond to changes in the environment and thus affect sex. Although DNA methylation was unfound directly participated in the regulation of the sex determination pathway, sex determination may be involved in other epigenetic factors, such as histone modification, which has been noticed in turtles (Ge et al., 2018). So, conserved master genes in vertebrates sex determination pathway are not utilized in oysters through DNA methylation, and methylated housekeeping genes may be trigger sex differentiation in the process of oysters' sex determination.

Organisms whose sex is determined by the cooperators of genetics and environment can regulate gonadal development through epigenetic responses to environmental factors, especially temperature and food (Anastasiadi et al., 2018; Bonasio et al., 2012; Lyko et al., 2010; Navarro-Martín et al., 2011; Shao et al., 2014). The response to environmental signals is concerned with protein kinase C (PKC), which can alter the expression of sex determination related genes and sex phenotype (Jiang et al., 2016; Koji et al., 2006; Toyota et al., 2017). The activity of PKC is regulated by DGKD, which exchanges diacylglycerol into phosphatidic (Sakane et al., 2002; Sato et al., 2013). Methylation analysis of individual genes showed that *Dgkd* was methylated in the whole gene region, and that the promoter region showed significant methylation differences between males and females. In addition to gene body methylation involved in transcription, the methylation of promoter in development genes has a direct influence on their expression (Saint-Carlier and Riviere, 2015). Methylation in the *Dgkd* promoter was negatively correlated with its expression, suggesting that the promoter methylation, which was well-known to regulate gene expression in mammals and plants (Feng et al., 2010; Weber et al., 2007; Zhang, 2008; Zhang et al., 2019), is also functional in the expression of genes in oysters. Therefore, *Dgkd* may be a potential sex differentiated gene that regulates downstream genes through DNA methylation in response to environmental signals.

The sexual phenotype of an oyster individual without external sexual characteristics is unaware unless it is sacrificed for histological analysis. Therefore, effective molecular markers that can predict the direction of sex differentiation at resting stage are of great value for the study of sex differentiation. Epigenetic markers have been reported to predict sex in fish with an accuracy of ~90% (Anastasiadi et al., 2018). Our study suggests that *Dmrt*, *Insr* and *Stpg2* could not be utilized as epigenetic markers for small differences of methylation levels. Although marked methylation difference was displayed in *Vg* between males and females, the low accuracy of sex prediction also cannot be applied as an epigenetic marker in oysters. Interestingly, the DNA methylation modifications in *Dgkd* can be used as a molecular marker to distinguish males for individuals with MSP specific bands of *Dgkd* were all males. Since the presence of transient methylation (Roberts and Gavery, 2012), whether this epigenetic marker is useful in resting stage is uncertain. However, our results show that the correlation can be established between epigenetic markers of mature gonads and sexual phenotype, which provides reference data for studying epigenetic marker of other gonadal development stages, especially the resting period.

5. Conclusions

In order to characterize epigenetic mechanisms in sex determination of oysters, the methylome data of male and female gonads were analyzed. Our results revealed that methylation was indirectly involved in the regulation of sex differentiation pathway and the master genes in the vertebrate sex determination pathway were not utilized by DNA methylation in oysters. In addition, genes in the conserved signaling pathways, rather than the key master genes in the sex determination pathway, were the major targets of substantial DNA methylation modification. DNA methylation fine-tuned housekeeping genes to regulate gene expression and sex differentiation. In addition, *Dgkd* methylation could serve as a potential epigenetic marker to identify sex in oysters. Our research provides a new insight into the sex determination mechanisms of DNA methylation in invertebrates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146260.

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