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—Original Article—

The Putative Promoters of Germ Cell-specific Genes and *Nanog* are Hypomethylated in Chicken Sperm

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Abstract. Germ cell-specific genes such as *Ddx4*, *Dnd1*, and *Dazl* play critical roles in the proliferation and survival of germ cells. However, the methylation state of the promoter in mature germ cells is still unknown. Here, we investigated the methylation levels of these genes and the pluripotency marker gene *Nanog* in chicken sperm as compared with the *Alb* gene in the liver. CpG islands and/or promoter motifs such as TATA box, GC box and CAAT box were found within the putative promoter regions that we identified. By using the bisulfite reaction, CpG sites in the putative promoters were converted, and they were analyzed by sequencing. The putative promoters of *Ddx4*, *Dnd1*, *Dazl* and *Nanog* showed very low methylation levels in sperm, but they were highly methylated in the liver. Conversely, the *Alb* gene promoter was highly methylated in sperm and hypomethylated in the liver. However, no transcripts of *Ddx4*, *Dnd1*, *Dazl* and *Nanog* were detected in sperm or the liver. Also, no transcripts of *Dnmt1* and *Dnmt3a* were detected in sperm. Our present results may indicate that these germ cell-specific genes and the pluripotency marker gene are ready to express any time after fertilization. Our findings showing that low methylation and selective DNA methylation of specific genes are present in chicken sperm contribute to our understanding of fertilization and embryogenesis of birds.

Key words: CpG site, DNA methylation, Gene expression, Germ cell-specific genes, Sperm

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DNA methylation occurs at cytosine-phosphodiester-guanine sites (CpG sites), and its patterns are established and maintained during cell differentiation and cell division by DNA methyltransferases (DNMTs). In primordial germ cells (PGCs), the genome-wide methylation state inherited from parents is erased in a certain period, and new methylation patterns are established [1, 2]. The relationship between methylation and the regulation of imprinted genes in germ cells has recently attracted considerable attention [3–7]. In mammals, the methylation levels of maternal and paternal imprinted genes are different between oogenesis and spermatogenesis [8].

During gametogenesis in chickens, several germ line-specific genes such as *Ddx4* [9], *Dnd1* [10], and *Dazl* [11] are expressed in germ cells. These proteins play critical roles in the survival and differentiation of germ cells. In *Xenopus laevis*, PGCs are not generated after injection of the anti-*Xenopus* Ddx4 homologue antibody into tadpoles [12]. In mice, a defect in Ddx4 function results in the failure of differentiation and the promotion of apoptotic cell death [13]. The *Ter* mutation in the *Dnd1* gene is known to cause marked decreases in mouse PGCs [14]. In *Dazl*-deficient mice, germ cells do not initiate meiosis [15].

Some studies have reported that the DNA methylation in gene promoters prevents the gene transcription by inhibiting the binding of transcription factors to some promoters [16, 17]. Although the methylation of imprinting genes plays an important role in sex determination, at the same time, that of germ cell-specific genes is also important for germ cell survival. In humans, incorrect DNA methylation of the *DAZL* promoter regions of sperm, identified by the presence of CpG islands, causes abnormal spermatogenesis [18]. Although the CpG island is one of the guideposts of the promoter, all promoters do not always have a CpG island. In mammals, the ratio of genes having tCpG islands around the promoter is approximately 40%. Imprinting genes are methylated with the progression of differentiation of cells, including germ cells, while a non-imprinting gene, *Brahma*, displays a different methylation pattern during spermatogenesis and oogenesis [19, 20]. Based on these differences in gene methylation pattern among the sexes and genes, there is a certain mechanism in mature germ cells for expression of several genes immediately after fertilization. In the zygote during cleavage, the maternal transcription factor is important for embryogenesis [21, 22]. In vertebrates including chickens, the precursors of germ cells arise in the early stage of embryogenesis. One of the germ cell-specific genes, *vas*, is expressed soon after fertilization in germ cells, despite the fact that the general RNA synthesis is not detected soon after fertilization in drosophila [23]. This indicates the importance of the expression of germ cell-specific genes supporting germ cell survival.

During spermatogenesis, histones are replaced by testis-specific histone variants in male germ cells. Then the variants are converted to transition proteins, which are replaced with protamine in the condensing chromatin during elongating spermatid stage in humans [24]. As a result, the gene expression activity is diminished. Based

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on this inactivity of transcription, in sperm, it is considered that there is an epigenetic mechanism that makes gene expression easier in the zygote. However, the methylation pattern of germ cell-specific genes in mature germ cells remains unknown. Thus, in the present study, we focused on the putative promoter region of germ cell-specific genes and the pluripotency marker gene *Nanog*. We first analyzed the methylation states of these genes in sperm.

Materials and Methods

Chicken samples

Chicken sperm and livers were collected from White Leghorn chickens. The semen was separated into 90% and 45% layered Percoll solutions (MP Biomedicals, Solon, OH, USA) by centrifuging for 15 min at 1000 *g* and 4 C. All procedures were performed under the control of the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and in compliance with Law No. 105 and Notification No. 6 of the Government of Japan.

Genomic DNA extraction and bisulfite reaction

Genomic DNA was isolated using an AllPrep DNA/RNA Micro Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Unmethylated cytosine in the genomic DNAs isolated from each sample was converted to uracil using a MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures Pty, Randwick, Australia) according to the manufacturer's protocol.

Genomic DNA subcloning and sequencing

Genomic DNA sequences, CpG islands, and transcription factor binding sites were identified using the NCBI Entrez database (http://www.ncbi.nlm.nih.gov/Entrez/), MethPrimer (http://www.urogene.org/methprimer/), and TFBIND (http://tfbind.hgc.jp/), respectively. The target sequences were isolated from the genomic DNAs treated with the bisulfite reaction by PCR. The primer sets used for the PCR reaction are listed in Supplemental Table 1 (on-line only). The PCR reaction was performed in a total volume of 10 µl containing 40 ng genomic DNA, 1 µl of 10 × PCR buffer, 0.2 mM of dNTP, 0.25 units TaKaRa Taq HS (Takara Bio, Otsu, Japan) and 250 nM of each specific primer. The target sequences were ligated with T-Vector pMD20 (Takara Bio) using a DNA Ligation Kit, Mighty Mix (Takara Bio), and the construct was transformed into *E. coli* HST08 (Takara Bio) for sequencing.

Total RNA extraction and reverse transcription

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase (Wako, Osaka, Japan). cDNA was synthesized with oligo $(dT)_{15}$ and random primers using a GoTaq 2-Step RT-qPCR System (Promega, Madison, WI, USA).

RT-PCR

RT-PCR was performed in a 10-µl volume containing 45 ng cDNA, 1 µl of 10 × PCR buffer, 0.2 mM of dNTP, 0.25 units TaKaRa Taq HS (Takara Bio), and 250 nM of each specific primer (Supplemental Table 2: on-line only). All primer pairs were designed to span introns to prevent amplification of products from genomic DNA. PCR products were electrophoresed using 1.5% agarose gel and were detected by Midori Green DNA Stain (Nippon Genetics, Tokyo, Japan).

Results

Searching for promoter motifs upstream of the first exon in the chicken

Since the chicken genomic database still does not contain sufficient data to identify the promoter, we searched for promoter motifs such as TATA box, GC box, and CAAT box upstream of their first exon as compared with the mouse genomic database. The structures of chicken *Ddx4*, *Dnd1*, *Dazl* and *Nanog* were investigated. The *Alb* gene was used as a reference. Since each chicken gene was constructed by several exons, we focused on a region upstream of the first exon of each gene as a putative promoter. A CpG island was present near the first exon of *Dnd1* and *Dazl* in the chicken. Chicken *Ddx4, Nanog* and *Alb* lack CpG islands within the investigated region. However, highly homologous sequences of TATA box, GC box, and CAAT box were located near the first exon of each chicken gene (Fig. 1). Consequently, the region approximately 500 bp upstream from the first exon may function as a promoter.

Expression of DNA methyltransferases in sperm and the liver

To further confirm whether the methylation level results on the expression levels of the Dnmt family, the transcripts of *Dnmt1*, *Dnmt3a* and *Dnmt3b* were analyzed in sperm and liver samples. As shown in Fig. 2, the transcripts of *Dnmt1* and *Dnmt3a* were detected in liver samples, but not in sperm.

Identification of methylated CpG sites in the putative promoters

To identify the number of CpG sites in the putative promoters of *Ddx4*, *Dnd1*, *Dazl*, *Nanog* and *Alb*, we searched for the contiguous sequence of cytosine and guanine in the region 500 bp upstream from the first exon. The results are shown in Fig. 3A. The number of CpG sites was diverse among these five genes, and one CpG island existed in the 5′UTR of *Dnd1* (–168 bp to –324 bp) and *Dazl* (–185 bp to –388 bp). Genomic DNA samples were prepared from sperm and liver samples to analyze their methylation states. After applying the bisulfite reaction to the target sequences, 12 clones were obtained from each gene. The methylation levels of CpG sites in all genes examined were very low in sperm samples, while *Alb* was highly methylated. Conversely, most CpG sites in *Ddx4*, *Dnd1*, *Dazl* and *Nanog* were highly methylated in liver samples, while the methylation level of *Alb* was low (Fig. 3B, Table 1).

Expression of the target genes in sperm and the liver

To further investigate the relationship between gene transcription and methylation in sperm and the liver, mRNA samples were subjected to RT-PCR. No transcripts of *Ddx4*, *Dnd1*, *Dazl* or *Nanog* were detected in either sperm or liver samples (Fig. 4). However, the transcript of liver *Alb*, which was hypomethylated in the putative promoter, was detected in liver samples.

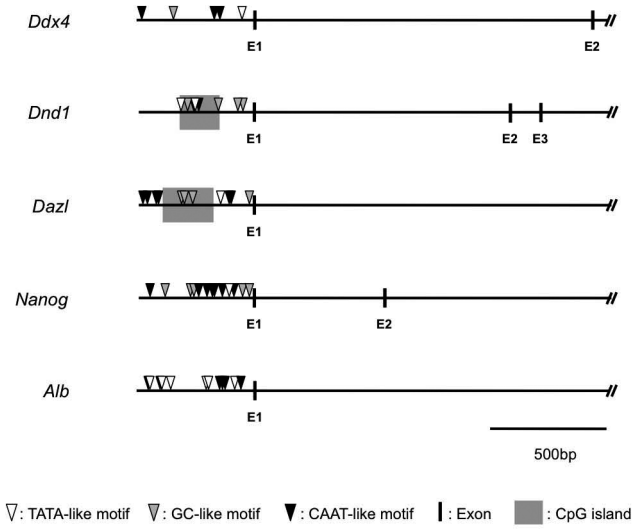

Fig. 1. Structure of *Ddx4*, *Dnd1*, *Dazl*, *Nanog*, and *Alb* in the 2000-bp region including the downstream regions of the putative promoter regions in the chicken. Vertical line: exon. Shadow: CpG island. White triangle: TATA and TATA-like motifs. Gray triangle: GC and GC-like motifs. Black triangle: CAAT and CAAT-like motifs.

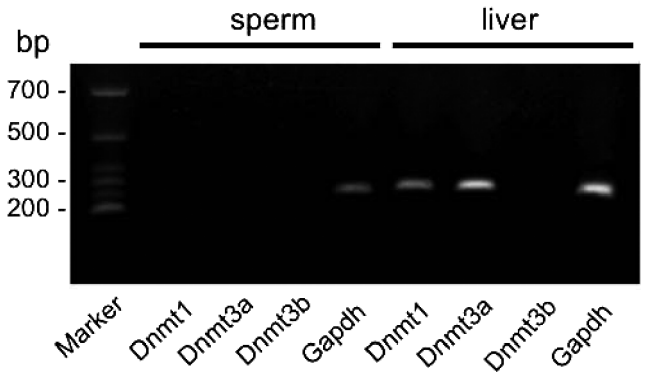

Fig. 2. Analyses of the expression of *Dnmts* in chicken sperm and liver samples. Sperm and liver mRNA samples were subjected to RT-PCR for expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b*. *Gapdh* was used as an internal control.

Discussion

Germ cell-specific genes such as *Ddx4*, *Dnd1*, and *Dazl* play critical roles in the proliferation and survival of germ cells. These proteins are all classified in the RNA binding protein. Ddx4 plays a role in germ cell proliferation as meiotic progression [25]. Dnd1 is associated with germ cell survival and the mitotic arrest of male PGCs [26, 27]. *Dazl*-defective mice are infertile and exhibit a decrease in germ cells [15]. In oligoasthenoteratozoospermic men, incorrect methylation in the *Dazl* promoter is a cause of infertility [18]. In the chicken, the expression of the germ cell-specific proteins dynamically changes during germ cell differentiation [11]. This suggests that the expression of genes coding germ cell-specific proteins is also changed during gametogenesis by a certain mechanism. Epigenetic mechanisms contribute to the regulation of imprinted gene transcription [28–31]. Methylation at CpG sites plays a principal role in gene silencing [32–37]. In the present study, we investigated the methylation states of putative promoter regions in non-imprinted germ cell-specific genes. The genes *Ddx4*, *Dnd1*, *Dazl* and *Nanog* are different in terms of their numbers of CpG sites within the 500–bp putative promoters. Specifically, *Dnd1*, *Dazl* and *Nanog* have many CpG sites, while *Ddx4* has only one. In a recent study using mouse spermatogenic cells, it was reported that the number of CpG sites affects the silencing of intronless genes [38]. The fact that the methylation site of the *Ddx4* promoter region is only slightly silenced may indicate that the Ddx4 protein is expressed nearly until terminal differentiation of the germ cells.

Certain motifs such as TATA box, GC box and CAAT box appear in promoter regions of eukaryotes. Transcriptions of most genes including imprinted genes are controlled by these elements and epigenetic mechanisms [39–44]. At the TATA box, TFIIB and TFIIF, the basic transcription factors, promote gene transcription. One of the well-known transcriptional factors, Sp1, promotes gene transcription via its binding to the GC box in the promoter [45]. DNA methylation can potentially inhibit the binding of transcription factors and repress gene transcription [46]. The methylation patterns of some genes are different among organisms and between sexes. In mice, methylation in the promoter of *Brahma* decreases during spermatogenesis but increases during oocyte maturation [20]. In the present study, we identified the highly homologous promoter motifs including TATA box. The presence of the highly homologous TATA box suggests the possibility of the combination of basic transcription factors, and the region works as a promoter.

In contrast to the histone acetylation involved in histone acetyl-transferase and histone deacetylase [47–49], DNA methylation is a strict modification with a process that is regulated by the Dnmt family, including Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L. Of the Dnmt family, Dnmt3L is lacking in chickens [50]. Dnmt1 maintains DNA methylation patterns from parental cells to daughter cells [51–53]. Dnmt3a and Dnmt3b are involved in *de novo* methylation, and they work during early development and gametogenesis in mammals [54, 55]. *De novo* methylation occurs primarily in germ cells and pluripotent cells for the establishment of individual methylation patterns [56]. The above studies strongly suggest a relationship between methylation of the promoter and the expression of Dnmts in germ cells. In the present study, transcripts of *Dnmt1* and *Dnmt3a*, but not *Dnmt3b*, were detected in liver samples but were not expressed in sperm. The lack of gene expression of *Dnmts* in sperm indicates that the activity of the DNA methylation is low in sperm, while it is high in the liver. We also found that methylation levels in the putative promoters of germ cell-specific genes and *Nanog* were low in sperm, indicating that these genes may be easily expressed in sperm. However, no transcripts were detected in sperm. Conversely, it is very reasonable that the putative promoters of germ cell-specific genes are highly methylated in the liver, where they are not expressed. Interestingly, the methylation level of the *Alb* promoter was very low in liver samples, where the gene is constantly expressed, while

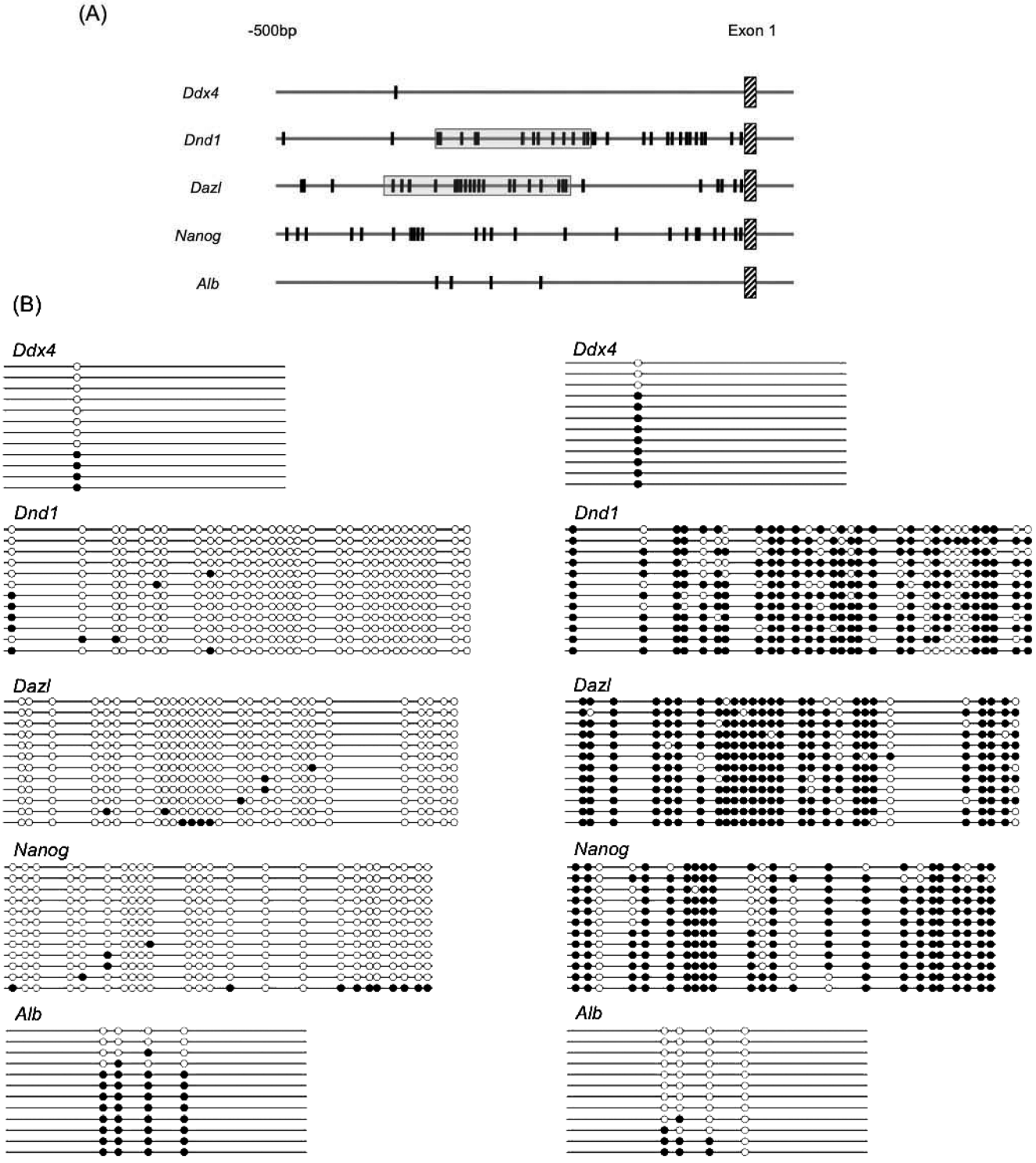

Fig. 3. Methylation of CpG sites within the putative promoter region containing the region 500 bp upstream from the first exon. (A) Localization of CpG sites and a CpG island within the putative promoter region. *Ddx4*, *Nanog* and *Alb* lack CpG islands. Vertical line: CpG site. Shadow: CpG island. (B) Methylated CpG sites in the putative promoter regions of *Ddx4*, *Dnd1*, *Dazl*, *Nanog* and *Alb* in chicken sperm and liver samples. Left: sperm; right: liver. Filled circles and open circles represent the methylated and unmethylated CpG sites, respectively. Each line represents an examined clone. The localizations of the CpG sites in each gene shown in A (vertical line) and B (circle) represent the same positions.

Table 1. Methylation levels in the promoters of the germ cell-specific genes, the pluripotency marker gene and the albumin gene in chicken sperm and liver samples

Genes	No. of CpG sites	Methylation level (%)	
		Sperm	Liver
Ddx4	1	33	75
Dnd1	30	3	75
Dazl	28	3	88
Nanog	24	5	82
Alb	4	71	17

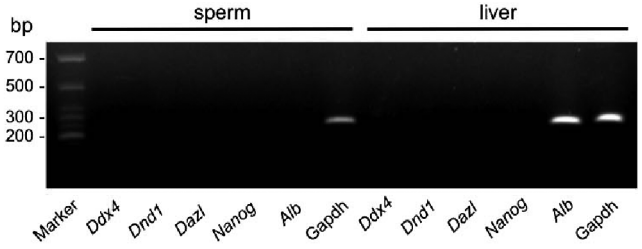

Fig. 4. Analyses of the expression of *Ddx4*, *Dnd1*, *Dazl*, *Nanog* and *Alb* in chicken sperm and liver samples. Sperm and liver mRNA samples were subjected to RT-PCR for expression of *Ddx4*, *Dnd1*, *Dazl*, *Nanog* and *Alb*. *Gapdh* was used as an internal control.

it was very high in sperm. Thus, genes are selectively methylated by unidentified mechanisms. Our present study supports a recent report demonstrating that the promoter CpG islands of germ cell-specific genes are hypermethylated in mouse somatic tissues [57].

One of the prospective reasons for this selectivity is that the genes having a hypomethylated putative promoter are necessary for the development of germ cells and embryos in early embryogenesis. Some studies have indicated the importance of the maternal factors stored in the egg for the zygotic gene activation in zygotes [21, 22]. Additionally, in the embryo of most vertebrates, the precursors of germ cells arise soon after fertilization [9, 23, 58]. Thus, since germ-cell specific genes such as *Ddx4*, *Dnd1* and *Dazl* and pluripotency marker genes such as *Nanog* and Oct-4 need to be expressed soon, these genes might be maintained in a state with a low methylation level in their promoters.

In the process of producing sperm and eggs, the germ cells differentiate several times and undergo meiosis. This is the most distinctive event for germ cells, and many genes are involved in this process. Elucidation of the relationship between methylation and expression of functional genes is important for clarification of the mechanism of reproduction. In conclusion, the present study provides novel evidence that the putative promoters of the germ cell-specific genes *Ddx4*, *Dnd1* and *Dazl* and the pluripotent marker gene *Nanog* are hypomethylated in chicken sperm. These genes, which have critical roles in the proliferation and survival of germ cells and the pluripotency of embryonic cells, may be ready to be expressed soon after fertilization. Conversely, the promoter of the *Alb* gene, which is not essential for their proliferation and survival, is highly methylated in chicken sperm. Our findings showing that low methylation and selective DNA methylation of specific genes are present in chicken sperm contribute to our understanding of fertilization and embryogenesis of birds, but the molecular mechanism(s) remains to be clarified in the future.

Conflict of interest: The authors declare that no conflicts of interest exist that would prejudice the impartiality of this study.

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