

—Original Article—

## DNA methylation-mediated transcription factors regulate *Piwil1* expression during chicken spermatogenesis

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**Abstract.** The P-element induced wimpy testis (Piwi) protein family is responsible for initiating spermatogenesis and maintaining the integrity of germ cells and stem cells, but little is known regarding its transcriptional regulation in poultry. Here, we characterized the methylation status of the *Piwil1* promoter in five different spermatogenic cell lines using direct bisulfite pyrosequencing and determined that methylation correlates negatively with germ cell type-specific expression patterns of *piwil1*. We demonstrated that methylation of the -148 CpG site, which is the predicted binding site for the transcription factors TCF3 and NRF1, was differentially methylated in different spermatogenic cells. This site was completely methylated in PGCs (primordial germ cells), but was unmethylated in round spermatids. A similar result was obtained in the region from +121 to +139 CpG sites of the *Piwil1* promoter CpG island, which was predicted to contain SOX2 binding sites. In addition, demethylation assays further demonstrated that DNA methylation indeed regulates *Piwil1* expression during chicken spermatogenesis. Combined with transcription factor binding site prediction, we speculate that methylation influences the recruitment of corresponding transcription factors. Collectively, we show the negative correlation between promoter methylation and *piwil1* expression and that the spatiotemporal expression of chicken *Piwil1* from the PGC stage to the round spermatid stage is influenced by methylation-mediated transcription factor regulation.

**Key words:** Chicken, DNA methylation, *Piwil1*, Spermatogenesis

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**S**permatogenesis is a complex developmental program by which male primordial germ cells (PGCs) differentiate into round spermatids through mitosis and meiosis. To become mature and functional sperm, male germ stem cells gradually lose their stem cell potential and undergo differentiation. During differentiation and spermatogenesis, orderly and precise spatiotemporal regulation of the expression of a variety of genes is essential to specify the germ cell fate [1, 2].

Many studies have reported that the protein Piwi is critical for both initiating spermatogenesis [3, 4] and maintaining the integrity of germ cells and stem cells in mice, zebrafish, and chicken [5–7]. *Piwi* genes encode Piwi proteins, which comprise a subfamily of Argonaute proteins. Argonaute proteins are known as PAZ Piwi domain (PPD) proteins and are a well-conserved family consisting of two subclasses: Ago and Piwi. There are four members of related Piwi subfamily genes termed *Piwil1* (Piwi-like homolog 1), *Piwil2*, *Piwil3*, and *Piwil4*. The mouse genome encodes three members of the

Piwi subfamily, Miwi (*Piwil1*), Miwi2 (*Piwil4*), and Mili (*Piwil2*), which are involved in the regulation of spermatogenesis [6–8]. The *Drosophila* genome also encodes three members: Piwi, aubergine (Aub), and argonaute 3 (Ago3) [9] which are each necessary for self-renewal of germ-line stem cells. Zebrafish has only two members of the Piwi family, Ziwi (zebrafish piwi/*Piwil1*) and Zili (Ziwi-like/*Piwil2*), of which Ziwi is responsible for maintenance of the germ line [7]. The chicken Piwi-like protein 1 (Ciwi) and 2 (Cili) were identified in 2012 [10]. Each of these Piwi proteins contributes to different stages of reproductive development, necessitating the precise spatiotemporal expression of different genes. The process of transcriptional regulation of *Piwi* in poultry spermatogenesis is poorly understood.

In this study, we analyzed the methylation status of the chicken *Piwil1* promoter region in different spermatogenic cells. Furthermore, we used 5-aza-2'-deoxycytidine (5-aza) to inhibit DNA methyltransferase activity in PGCs in order to elucidate the requirements for methylation-mediated regulation of spatiotemporal expression of *Piwil1* during spermatogenesis. In doing this, we hoped to enhance our understanding of how testis-specific expression of *Piwil1* is regulated during chicken spermatogenesis.

### Materials and Methods

#### Ethics statement

All experimental procedures were performed in accordance with

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the Regulations on The Administration of Experimental Animals issued by the Ministry of Science and Technology in 2001 (Beijing, China). All the animal experiment protocols were approved and guided by the Animal Care and Use Committee of Yangzhou University.

#### *Cell isolation, culture, and identification*

Fertilized eggs from Rugao Yellow Chickens were provided by the Poultry Institute of the Chinese Academy of Agriculture Sciences and were incubated at 37.8°C and 70% relative humidity. PGCs were isolated from embryonic gonads at the Hamburger and Hamilton (H&H) Stage 27 (120 h post incubation) [11] under a stereomicroscope and then separated by density gradient equilibrium centrifugation and the differential adhesion method [12, 13]. Chicken spermatogonial stem cells (SSCs) were isolated following a previously published method [13] with slight modifications. Briefly, testes were aseptically dissected from 18-day-old embryos (H&H stage 44) and mechanically cut into small pieces using sterile surgical scissors. After digestion of testis tissue, SSCs were purified by density gradient centrifugation and the differential adhesion method. The different types of spermatogenic cells from testicular tissues were sorted by flow cytometry (FACS Aria, BD Biosciences, San Jose, CA, USA) [14, 15]. Cells were dyed with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Beyotime Biotechnology, Shanghai, China) prior to flow cytometry to identify the haploid, diploid, and tetraploid cells according to the intensity of DNA staining. Haploid cells were round spermatids, diploid cells were spermatogonia, and tetraploid cells (4n) were primary spermatocytes that had completed DNA replication but not division during meiosis I. Until the separation and identification of different cells, PGCs and SSCs were cultured in knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 2.5% chicken serum (Gibco), 2 mM L-glutamine (Gibco), 1× MEM nonessential amino acids (WISENT, St. Bruno, Quebec, Canada), 2 mM sodium pyruvate (Gibco), 1× HEPES (WISENT), 0.1 mM β-mercaptoethanol (Sigma Aldrich, St Louis, MO, USA), 5 ng/ml human stem cell factor (hSCF) (Sigma Aldrich), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma Aldrich), and 10 ng/ml mouse leukemia inhibitory factor (Sigma Aldrich). The cells were cultured in an incubator at 37°C with 5% atmospheric CO<sub>2</sub> and 60–70% relative humidity.

PGC colonies were identified using the periodic acid-Schiff [16] stain (Jiancheng, Nanjing, China) and stage-specific embryonic antigen-1 (SSEA-1) staining (Santa Cruz, 100 Dallas, TX, USA); SSCs were identified using SSEA-1 staining according to the instructions provided.

#### *DNA extraction and bisulfite conversion*

Genomic DNA from different types of spermatogenic cells was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA quality and concentration were assessed with a Nanodrop-1000 spectrophotometer (Thermo Scientific, MA, USA). Bisulfite conversion of DNA was performed with the EpiTect Bisulfite Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions for bisulfite pyrosequencing (BP) analyses (50 ng DNA/sample).

#### *Bioinformatics analyses*

CpG island predictions for the chicken *Piwill* promoter were made with the MethPrimer software (<http://www.urogene.org/methprimer/index1.html>) using the following parameters: island size > 200, GC% > 50.0%, and Obs/Exp > 0.6. The JASPAR software ([http://jaspar.genereg.net/cgi-bin/jaspar\\_db.pl](http://jaspar.genereg.net/cgi-bin/jaspar_db.pl)) was used to predict transcription factor binding sites in the chicken *Piwill* promoter region using the JASPAR CORE Vertebrata database and a profile score threshold of ≥ 85%.

#### *Direct bisulfite pyrosequencing*

Bisulfite converted DNA was eluted in 20 μl of elution buffer and was subjected to polymerase chain reaction (PCR) amplification using primer sets targeting the specific region of the *Piwill* promoter. Four primer sets (Table 1) were designed to amplify four different domains of the *Piwill* promoter using the PyroMark Assay Design software (PSQ assay design software) (Qiagen). The PCR product was confirmed by 1.5% agarose gel electrophoresis to determine the size and quality of the products and to eliminate the possibility of primer dimers. The specific PCR products were then subjected to quantitative pyrosequencing analysis using a PyroMark Gold Q96 system (Qiagen) according to the manufacturer's instructions. The results were analyzed with the PyroMark CpG software (Qiagen).

#### *5-aza-2'-deoxycytidine treatment*

To inhibit DNA-methyltransferase, PGCs were seeded in 12-well plates and allowed to attach for 24 h. The cells were then treated with or without 5-aza-2'-deoxycytidine (Sigma Aldrich) at a concentration of 0.05 μM and 0.08 μM for 24 h, as described under the section "Cell isolation, culture, and identification". At 24 h, the cells were harvested and counted. Cell viability was assessed using a CCK-8 assay (Vazyme Biotech, Piscataway, USA), which is a modified method based on the classical MTT reduction assay [17]. Two biological replicates were performed with three technical replicates each.

#### *RNA isolation and qRT-PCR analysis*

Total RNA was extracted from different spermatogenic cells and PGCs treated with or without 5-aza at 0.05 μM, using TRIzol reagent [18] and quantified with a Nanodrop-1000 spectrophotometer. The RNA (1 μg) isolated for each condition was reverse transcribed to complementary DNA (cDNA) using the FastQuant RT Kit (with gDNase) (Tiangen, Beijing, China) following the manufacturer's instructions. The cDNA was stored at -20°C until required. Quantitative real time PCR (qRT-PCR) was performed for *Piwill* and gene expression was normalized against *GAPDH*. The primers were designed with the Oligo7.0 software according to the sequences included in the GenBank database and were synthesized by the Shanghai Sangon Biotechnology (Shanghai, China) (Table 2). The experiment was performed on an ABI 7500 Real-time PCR Detection System (Applied Biosystems, Carlsbad, CA) using the UltraSYBR Mixture (with ROX) (CoWin Biotechnology, Beijing, China), and each sample was analyzed in triplicate.

#### *Statistical analyses*

All results are expressed as mean values. Statistical analyses and graphical representations were performed with the GraphPad

**Table 1.** Primers sets used for amplification of four *Piwill* promoter specific regions

Primer names	Primer sequences (5'–3')	Amplicon length (bp)	Annealing temperature (°C)
PIWIL1-1	F: Btn-GTAAGATTTGGAGAGAGAATGGAATTTAT R: CCCTACCAAACCTCTCTTCAACCTACAC S: AGGAAAATTTTTTATAAAAAGTTA	279 (–263~+15)	58
PIWIL1-2	F: Btn-AAGATTTGGAGAGAGAATGGAATTTTA R: CCCTACCAAACCTCTCTTCAACCTACAC S: AAAACCCACCAAACCTCTAATT	277 (–261~+15)	58
PIWIL1-3	F: Btn-GGTTTTAGGGAGTTAATTAGAGGG R: CCCTCAACATTACCTCAATCAC S: CTACCAAACCTCTCTTCAACCT	237 (–67~+169)	58
PIWIL1-4	F: Btn-GTGTAGGTTGAAGAGAGTTT R: ACCCTCAACATTACCTCAATC S: CAACATTACCTCAATCAC	182 (–11~+170)	58

F, forward primer; R, reverse primer; S, sequencing primer.

**Table 2.** Primers sets used for qRT-PCR

Gene symbol	Primer sequences (5'–3')	Amplicon length (bp)	Annealing temperature (°C)
<i>PIWIL1</i>	F: TCACCTGAGCAAAGACAAC R: TCCCGTAAAGGACAGTAAG	126	60
<i>GAPDH</i>	F: CGATCTGAACCTACATGGTTTAC R: 5'TCTGCCCATTTGATGTTGC	151	60

F, forward primer; R, reverse primer.

Prism 6 software (San Diego, CA). A P-value less than 0.05 was considered as significant and a P-value less than 0.01 was considered as extremely significant. The statistical tests used are indicated in the figure legends.

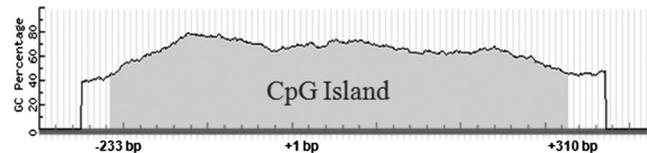
## Results

### *CpG island prediction*

To determine whether DNA methylation influences *Piwill* expression and regulation during chicken spermatogenesis, we first performed a CpG island prediction analysis in the 2 kb region upstream of the *Piwill* start codon revealing a large CpG island (543 bp, –233 to +310 bp, TSS = +1) in the 5'-flanking region of *Piwill* (Fig. 1).

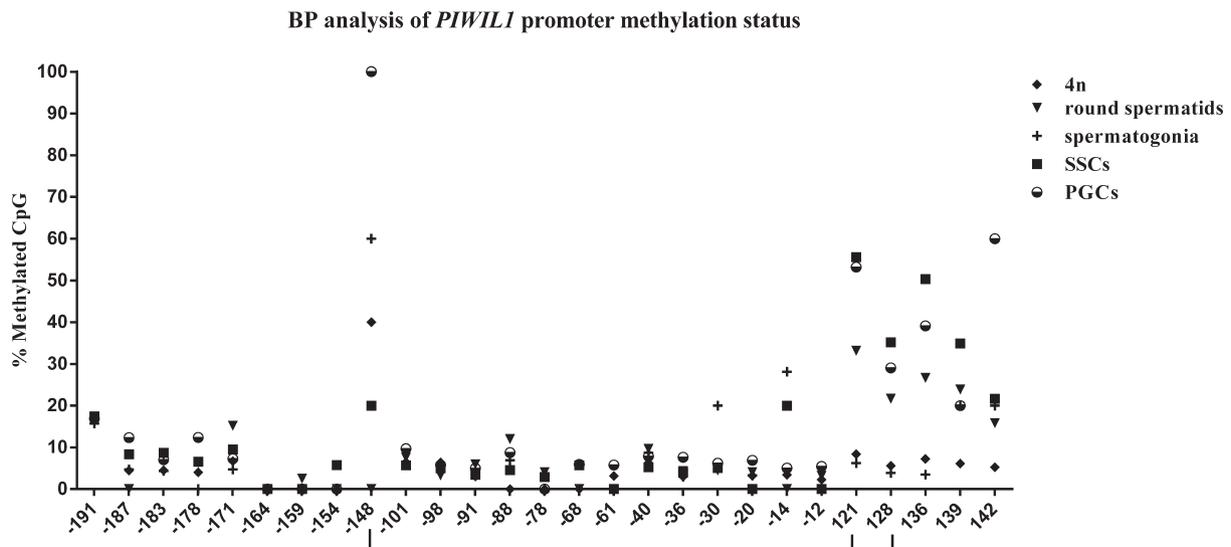
### *Promoter methylation status of *Piwill1* promoter in different spermatogenic cell types*

To determine the spatial and temporal methylation status of the predicted CpG island in the *Piwill* promoter region and its association with the expression and regulation of chicken *Piwill* during spermatogenesis, we analyzed bisulfite-treated genomic DNA isolated from different spermatogenic cells, including PGCs, SSCs, spermatogonia, 4n cells, and round spermatids. The mean methylation values of these CpG sites showed striking differences among different spermatogenic cells (16.21% in PGCs, 12.44% in SSCs, 8.42% in spermatogonia, 8.48% in round spermatids, and 5.62% in 4n cells).

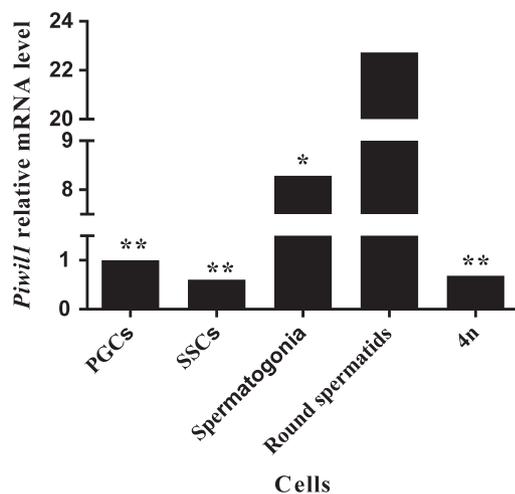


**Fig. 1.** CpG island (543 bp, –233 to +310 bp, TSS = +1) found in the 2-kb region upstream of *Piwill* ATG.

The mean methylation values of the *Piwill* promoter CpG island in five different types of spermatogenic cell populations are shown in Fig. 2. Our assays indicated the decreasing methylation of this region as spermatogenesis progressed. To compare this with the expression of *Piwill*, we measured *Piwill* mRNA expression in each of the cell types mentioned. *Piwill* was highly expressed in round spermatids whereas its expression in PGCs and SSCs was low (Fig. 3). These results indicate that *Piwill* expression and *Piwill* promoter methylation are inversely correlated in chicken spermatogenic cells. The CpG sites from +121 to +139 of the *Piwill* promoter CpG island in almost all spermatogenic cells were highly methylated except those in 4n cells and spermatogonia. The +121 and +128 CpG sites showed a similar methylation pattern across cell types: high methylation in SSCs and PGCs and low methylation in 4n and spermatogonia.



**Fig. 2.** Bisulfite pyrosequencing (BP) analysis of *Piwill* promoter methylation status in different spermatogenic cells. The position of each CpG is indicated (TSS = +1). The binding region for the transcription factors TCF3 and NRF1 includes the -148 CpG site and the SOX2 (from +121 to +128 bp, CGTTTGTC) binding region includes the +121 and +128 CpG sites.



**Fig. 3.** Relative *Piwill* mRNA expression in different spermatogenic cells of chicken. \*  $P < 0.05$ ; \*\*  $P < 0.01$  compared with the expression level in round spermatids. PGCs: primordial germ cells; SSCs: spermatogonia stem cells; 4n: tetraploid cells.

Computational prediction of transcription factor binding sites in the *Piwill* promoter CpG island indicated a potential binding site for SOX2 (+121 to +128 bp, CGTTTGTC). In addition, the -148 site of the *Piwill* promoter region in spermatogenic cells exhibited CpG-specific methylation, where this site was highly methylated in PGCs, SSCs, spermatogonia and 4n (probability = 100%, 20%, 60% and 40%, respectively), but was not methylated (probability = 0%) in round spermatids. This site was predicted to be a putative binding site for the transcription factors TCF3 and NRF1.

#### *Effect of 5-aza-2'-deoxycytidine on cell proliferation and viability*

To evaluate the potential relationship between *Piwill* promoter methylation and expression, PGCs were treated with 5-aza. We first assessed the impact of 5-aza on cell viability and proliferation using a CCK-8 assay. As shown in Fig. 4A, 0.08  $\mu\text{M}$  5-aza significantly decreased PGC viability ( $P < 0.05$  vs. untreated cells), whereas 0.05  $\mu\text{M}$  5-aza had no significant effect on cell viability ( $P = \text{NS}$ ). Similar results were observed for cell proliferation as well (Fig. 4B).

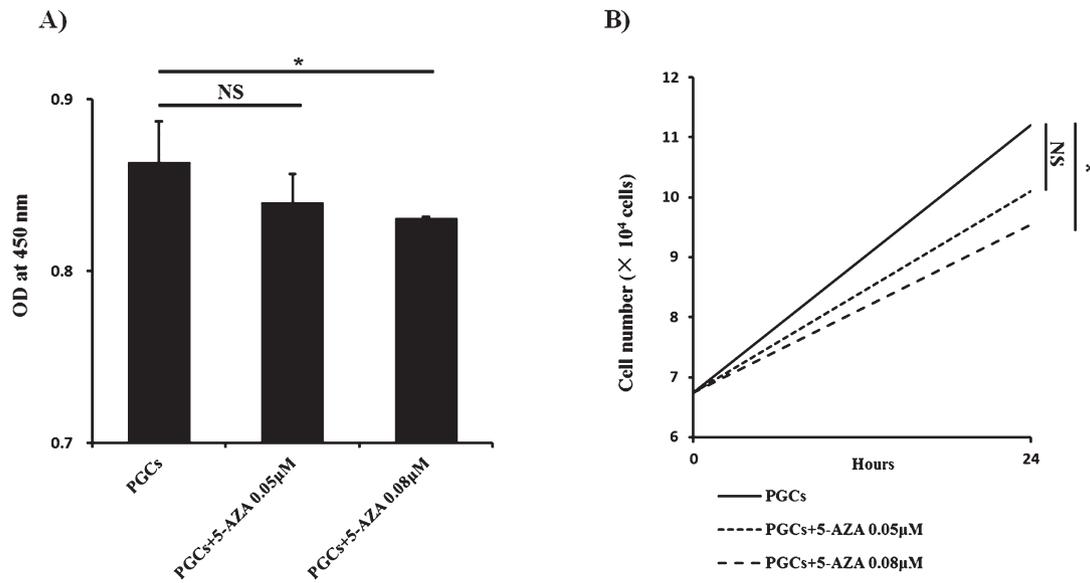
#### *Expression of Piwill after treatment with 5-aza-2'-deoxycytidine*

After assessing the influence of 5-aza on cell proliferation and viability, we treated PGCs with 0.05  $\mu\text{M}$  5-aza for 24 h and checked the *Piwill* mRNA expression level. As shown in Fig. 5, treatment with 5-aza induced nearly two-fold increase in *Piwill* mRNA expression compared to untreated cells ( $P < 0.05$ ).

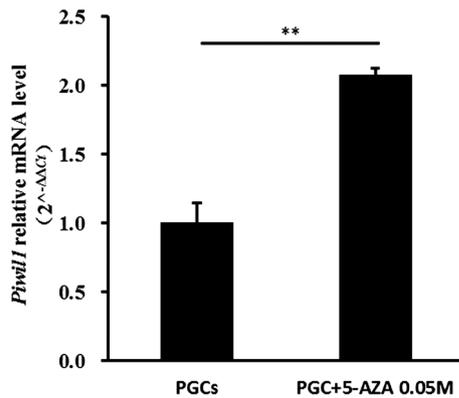
## Discussion

CpG islands are regions with enriched CG content and are usually associated with promoter regions. The methylation of one CpG dinucleotide within the transcriptional regulatory element of a promoter region can alter the accessibility of DNA to specific transcription factors, and thus cause gene silencing or decreased expression. In contrast, hypomethylation of CpG sites in the gene promoter regions is generally associated with increased gene expression.

In this study, we used direct bisulfite pyrosequencing to determine the methylation status of a predicted CpG island in the 5'-flanking region of the chicken *Piwill* promoter in different spermatogenic cells, including PGCs, SSCs, spermatogonia, 4n cells, and round spermatids. The results showed that the -148 CpG site and the CpG sites in the



**Fig. 4.** Effect of 5-aza on cell proliferation and viability. A) Cell viability was measured with the CCK-8 kit. NS, not significant; \*  $P < 0.05$  (Student-Newman-Keuls test) vs. untreated cells. B) Cell proliferation. The results are representative of 3 independent experiments.



**Fig. 5.** Impact of 5-aza on *Piwill* expression in PGCs. Results are representative of 2 independent experiments. \*\*  $P < 0.01$  (Student-Newman-Keuls test) vs. untreated cells.

posterior fraction of the *Piwill* promoter region CpG island were differentially methylated within different spermatogenic cells. The mean methylation degree of the CpG island in the *Piwill* promoter region was higher in PGCs and SSCs and lower in round spermatids (probability = 16.21%, 12.44%, and 8.48%, respectively), whereas *Piwill* was highly expressed in round spermatids and showed low expression in PGCs and SSCs. This suggests a negative correlation between methylation of the *Piwill* promoter and *Piwill* expression in chicken spermatogenic cells, which is similar to the reports by Chang *et al.* in the chicken and findings in other animals [5, 19]. Another finding of this study is that the -148 CpG methylation site containing predicted binding sites for the transcription factors TCF3

and NRF1 is differentially methylated in the spermatogenic cells types. PGCs showed complete methylation and round spermatids showed absence of methylation at this site, which suggests that TCF3 and NRF1 regulate *Piwill* expression in germ cells. The +121 and +128 CpG sites located in the predicted SOX2 binding region (+121 to +128 bp) were also differentially methylated, and showed a similar methylation trend, that is, higher methylation in PGCs and SSCs, and lower methylation in round spermatids. This, too, suggests that SOX2 might be involved in regulation of *Piwill* during chicken spermatogenesis.

To confirm that methylation affects the expression of *Piwill* in chicken spermatogenic cells, we used 5-aza to inhibit DNA methylation in chicken PGCs. When methylation was inhibited, the expression of *Piwill* in PGCs increased by nearly two-fold relative to that in untreated cells, further supporting the negative relationship between *Piwill* promoter methylation and *Piwill* expression. Methylation of CpG sites in the *Piwill* promoter might inhibit binding of the transcription factors TCF3 and NRF1, thereby repressing *Piwill* expression. Thus, when demethylation occurs, these transcription factors bind to the *Piwill* promoter and activate its expression.

To date, much of the research on the regulation of *Piwi* gene transcription has focused on the transcription factors NF-Y, USF, and A-MYB. Chang *et al.* and Sohn *et al.* reported that DNA methylation and NF-Y co-regulate *Piwill* expression in chickens [20, 21]. Hou *et al.* found that methylation-mediated USF controls *Miwi* expression from the midpachytene spermatocyte stage to the round spermatid stage in mice [5]. Li *et al.* found an evolutionarily conserved A-MYB feed-forward loop in which A-MYB regulates the expression of *Piwill* by binding its promoter in both rooster and mouse testis [22]. However, in this study, we discovered additional transcription factors that might be involved in regulating *Piwill* expression during chicken spermatogenesis. TCF3 was reported to be involved in mouse

embryonic stem cell self-renewal by repressing *Nanog* transcription [23–25]. NRF1 mediates the transcriptional regulation of key metabolic genes related to cellular growth and development [26–28]. Further studies on the mechanism of how these transcription factors regulate *Piwill* expression in chickens will provide a better understanding of spermatogenesis, germ-cell development, and differentiation.

### Acknowledgements

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