Animal Reproduction Science 162 (2015) 95–103

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

DNA methylation and NF-Y regulate *Piwil1* expression during chicken spermatogenesis

Guobin Chang[a,1], Rong Chen[a,b,1], Lu Xu[a], Teng Ma[a], Hongzhi Wang[a], Jing Chen[a], Yang Zhang[a], Zhiteng Li[a], Fang Wan[a], Xiaomin Guo[a], Qi Xu[a], Wenming Zhao[a], Guohong Chen[a,*]

[a] College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China
[b] Institute of Animal Science, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

ARTICLE INFO

Article history:
Received 13 July 2015
Received in revised form
22 September 2015
Accepted 26 September 2015
Available online 30 September 2015

Keywords:
Chicken
DNA methylation
*Pi*wil1
Spermatogenesis

ABSTRACT

The P-element induced wimpy testis (Piwi) protein family, a subfamily of the Argonaute protein family, is involved in gene silencing and shows specific expression in spermatogenic cells. To reveal the transcriptional regulatory mechanisms of *Piwil1* in chickens, we cloned sequences of the chicken *Piwil1* promoter region and performed luciferase reporter and electrophoretic mobility shift assays to analyze the transcriptional activity and identify important transcriptional regulatory elements. The results showed that the region from −90 to −43 in the 5′-flanking region of *Piwil1* contains a transcriptional regulatory CCAAT box that was necessary for the transcriptional activity of the *Piwil1* promoter. Moreover, the transcription factor nuclear factor Y (NF-Y) was bound to the *Piwil1* promoter CCAAT box specifically in germ cells. In addition, bisulfite sequencing to determine the methylation profile of the *Piwil1* promoter CpG island in different spermatogenic and non-germ cell populations was performed. Compared with germ cells, non-germ cells showed increased methylation of the promoter region containing the CCAAT box, loss of NF-Y binding, and silencing of the *Piwil1* locus. It is demonstrated that the specific expression of *Piwil1* in chicken germ cells is regulated by the transcription factor NF-Y and differential CpG island methylation.

1. Introduction

The P-element induced wimpy testis (*Piwi*) gene was first identified in the *Drosophila* ovary and was shown to regulate asymmetric division of germline stem cells (Lin and Spradling, 1997). The *Piwi* gene encodes the Piwi protein, which is a member of the Piwi subfamily of Argonaute proteins. The Piwi subfamily has three members in *Drosophila*-Piwi, aubergine (Aub), and argonaute 3 (Ago3) (Harris and Macdonald, 2001; Gunawardane et al., 2007), three in mice-Miwi/Piwil1 (Piwi-like 1), Mili/Piwil2, and Miwi2 (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004), four in human-Hiwi/Piwil1, Hili/Piwil2, Piwil3, and Hiwi2/Piwil4 (Sasaki et al., 2003), and two in zebrafish-Ziwi/Piwil1 and Zili/Piwil2 (Houwing et al., 2008). The Piwi proteins mainly associate with Piwi-interacting RNAs (piRNAs) (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006), and play

* Corresponding author.
E-mail addresses: passioncgb@163.com (G. Chang), chenrong_big@163.com (R. Chen), herry2800@163.com (L. Xu), 26177435@qq.com (T. Ma), 434373554@qq.com (H. Wang), 276532497@qq.com (J. Chen), zyang@yzu.edu.cn (Y. Zhang), 1169587200@qq.com (Z. Li), 583292863@qq.com (F. Wan), 1083866231@qq.com (X. Guo), 544460951@qq.com (Q. Xu), wmzhao@yzu.edu.cn (W. Zhao), ghchen@yzu.edu.cn (G. Chen).
[1] These authors have equal contribution to this work.

http://dx.doi.org/10.1016/j.anireprosci.2015.09.016
0378-4320/

an important role in germline development and gametogenesis. Similar to small interfering RNAs and microRNAs, piRNAs are small molecular RNAs that function in RNA interference. In humans, Piwi overexpression has been implicated in cancer (Qiao et al., 2002; Liu et al., 2006; Lee et al., 2006).

The different Piwi proteins play roles in different stages of the reproductive cycle. During mouse development, *Miwi2* is expressed from embryonic day 15.5 (E15.5) through the third day after birth (Carmell et al., 2007), *Mili* is expressed from E12.5 to the round spermatid stage (Unhavaithaya et al., 2009), and *Miwi* is expressed from the pachytene spermatocyte stage to the round spermatid stage (Deng and Lin, 2002). This precise temporal expression of different genes is often conferred by transcriptional regulation, and studies that analyze the promoters associated with these genes are important for understanding their transcriptional regulation (Sandelin et al., 2007; Trinklein et al., 2003; Kim et al., 2005). Currently, promoter studies include methods such as promoter cloning, promoter activity analysis, and promoter methylation analysis. In mice, there is a negative correlation between CpG island methylation in the *Miwi* promoter and specific expression of *Miwi* in germ cells (Hou et al., 2012). Furthermore, *Piwil1* expression in the testicles of the sterile male cattle yak is much lower than that of its parents (the yak and yellow cattle), whereas the methylation of the *Piwil1* promoter CpG island is much higher than that of its parents (Gu et al., 2013). Similarly, *Piwil1*, *Piwil2* and *Piwil4* expression levels in human orchioncus is much lower than those in normal testicles, whereas methylation of the CpG islands in the promoter regions of these genes is much higher than those in normal testicles (Ferreira et al., 2014). These studies revealed that the spatial and temporal expression of the *Piwi* genes is affected by methylation of their corresponding promoter regions.

There have been relatively few studies reporting on the *Piwi* genes in chickens. Kim et al. (2012) cloned the mRNA sequences of the chicken *Piwi* genes and showed, via RNA interference, that Piwi proteins in poultry have a conserved role in inhibiting transcriptional activity in germ cells. We previously showed that, in chicken, *Piwil1* expression is much higher in testicular tissues than in ovary, kidney and other tissues (Chen et al., 2013). In this study, we cloned sequences of the chicken *Piwil1* promoter region for the first time. Furthermore, we analyzed the transcriptional activity of these *Piwil1* promoter regions and methylation profiles of the *Piwil1* promoter CpG island in different spermatogenic cell populations, aiming to reveal the transcriptional regulatory mechanisms of *Piwil1* during chicken spermatogenesis.

2. Material and methods

2.1. Ethics statement

All of the animal experiments were reviewed and approved by the Academic Committee of the College of Animal Science and Technology, Yangzhou University according to the Management Measures of Laboratory Animal in Jiangsu Province (Permit Number: 45, Government of Jiangsu Province, China). All surgeries were performed according to the recommendations proposed by the European Commission (1997), and all efforts were made to minimize discomfort.

2.2. Sample collection and nucleic acid extraction

Testicular tissues were collected from ten adult Langshan chickens, snap frozen in liquid nitrogen, and stored at −80 °C. PGCs and SSCs were isolated from 90 chickens at the embryonic phase, H & H stage XIV–XVI and XLIV, respectively, and then were separated by density gradient equilibrium centrifugation and the differential adhesion method in our laboratory (Li et al., 2010; Yu et al., 2010). PGC colonies were identified using the periodic acid-Schiff (PAS) stain (Jiancheng, Nanjing, China) and a stage-specific embryonic antigen-1 (SSEA-1) stain (Santa Cruz, Dallas, TX, USA); SSCs were identified using SSEA-1 according to the instructions provided with each reagent. Spermatogonia and round spermatids were isolated from the testis and were sorted by flow cytometry (Mays-Hoopes et al., 1995; Mozdziak et al., 2006). GC-1 (mouse spermatogonial cell line) and COS-7 (African Green Monkey kidney fibroblast cell line) cells were purchased from the American Type Culture Collection (ATCC). CEF (chicken embryo fibroblast cell line) was stored in our laboratory.

For flow cytometry sorting, single-cell suspensions collected from testicular tissues were screened for PGCs and SSCs with the SSEA-1 antibody using a FACSAria flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and then the remaining cells were placed in 5-ml polystyrene round bottom test tubes and centrifuged. The supernatants were removed, and 1 ml of phosphate-buffered saline was added to the test tube to resuspend the cells. Next, 50 μl of 50 μg/ml propidium iodide (PI) (Sigma, Shanghai, China) labeling solution (with Triton X-100 (Sigma) as a solvent at a final concentration of 0.5 g/L) was added to label the DNA. The solution was placed at room temperature away from direct sunlight for 5 min. The labeled single-cell suspension was sorted using a FACSAria flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to separate and collect haploid and diploid cells for analysis.

Total RNA was extracted from testicular tissues and cells using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions. Genomic DNA was extracted from testicular tissues and cells using the Animal Tissue Genomic DNA Extraction Kit (Dingguo, Beijing, China), according to the manufacturer's instructions. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the concentration and purity ($OD_{260/280}$ ratio) of the total RNA and genomic DNA.

2.3. Rapid amplification of cDNA ends (RACE)

Total RNA from testicular tissue was used as a template to synthesize first strand cDNA using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). The gene-specific primers were designed according to the mRNA sequence (NM_001098852) of *Piwil1* in red