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Piwil1 mediates meiosis during spermatogenesis in chicken

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ABSTRACT

Piwil1 mediates spermatogenesis and ensures stable cell division rates in germline cells in mammals. However, the involvement of *Piwil1* in poultry spermatogenesis and meiosis is poorly understood. In the present study, we used TaqMan RT-qPCR to characterize *Piwil1* mRNA expression in different types of spermatogenic cells, including primordial germ cells (PGCs), spermatogonial stem cells (SSCs), spermatogonia cells (Sa), tetraploid cells (Tp), round sperm cells (Rs), mature sperm, and in PGCs treated with retinoic acid. Our results revealed that *Piwil1* is differentially expressed during spermatogenesis in chicken. Compared to PGCs, SSCs, Tp, and Sa, Rs cells presented the highest *Piwil1* mRNA expression levels. Retinoic acid significantly upregulated *Piwil1* and *Stra8* mRNA expression as well as *Piwil1* levels in chicken PGCs. In addition, retinoic acid induced PGCs to progress through all the meiotic stages, eventually leading to haploid cell formation, which was determined using flow cytometry and western blot analysis. Taken together, our results showed that during spermatogenesis, *Piwil1* was first expressed at low levels in germ stem cells, PGCs, and SSCs. Its expression levels increased during later meiosis stages. Finally, no expression was detected in mature sperm after meiosis. Treatment of PGCs with retinoic acid further demonstrated that *Piwil1* plays a key role in meiosis during chicken spermatogenesis.

1. Introduction

Spermatogenesis is a complex process that includes both mitosis and meiosis. Sperm formation depends on the expression and regulation of several genes at different stages, such as the P-element induced wimpy testis gene (*Piwi*). *Piwi* protein was first discovered in *Drosophila* germline cells (GSCs), and its function is to maintain testicular and ovarian development. Homology analysis showed that *Piwi* (particularly its C terminus) is homologous to *prg-1* and *prg-2* (*Piwi*-related gene) in nematodes, and to *Miwi*, *Mili*, and *Miwi2* in mice (Vourekas et al., 2012). In postnatal testis, *Mili* is expressed during the early stages of spermatogenesis apparently until the germ cells reach the meiotic pachytene stage, while *Miwi* is expressed from the pachytene to the haploid round spermatid stage (Vourekas et al., 2012). *Miwi* is very important for sperm formation and encodes a cytoplasmic protein that is specifically expressed in spermatocytes and sperm. *Miwi* functions not only in the self-regulation of cell molecules but also as a bridge, connecting trophoblast cells and the germline. *Piwi-like protein 1* (*Piwil1*) is a protein that in humans is encoded by the *Piwil1* gene (Cox et al., 1999; Sasaki et al., 2003). This gene encodes a member of the *Piwi* subfamily of Argonaute proteins, evolutionarily conserved proteins containing both *PAZ* and *Piwi* motifs that play important

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roles in stem cell self-renewal, RNA silencing, and translational regulation in diverse organisms. The encoded protein may play a role as an intrinsic regulator of the self-renewal capacity of germline and hematopoietic stem cells. If *Piwil1* is mutated in *Drosophila*, this results in division disorders in GSCs that eventually lead to infertility (Harris and Macdonald, 2001). To date, the function of *Piwil1* during poultry spermatogenesis has remained unclear. In the present study, we describe *Piwil1* mRNA expression patterns in different kinds of spermatogenic cells in order to clarify *Piwil1* function in poultry spermatogenesis and to characterize chicken azoospermia at the molecular level, further aiming to solve the decline of reproductive capacity in rooster by genetic approaches and to provide foundation on producing transgenic chickens by sperm-mediated gene transfer.

Retinoic Acid (RA) is a potent morphogen that has diverse effects during development and differentiation (Childs et al., 2011). It is secreted by mesonephric cells and plays a key role when germ cells enter meiosis. RA is abundant in the ovaries of mouse embryos, it allows cells to enter meiosis, and it has a specific gene expression (Bowles et al., 2006). Due to the action of the metabolizing enzyme *CYP26b1*, which is expressed in embryonic testis and which degrades RA, primordial germ cells (PGCs) do not receive the RA signal; consequently, the start of meiosis is inhibited in embryonic testis (Koubova et al., 2006). When the expression of *CYP26b1* declines sharply during sexual maturity, the RA signal enables the start of meiosis (Koubova et al., 2006). Furthermore, RA addition to cultured mouse embryonic testis in vitro can stimulate the XY germ cells to enter meiosis and compensate for endogenous RA degradation (Trautmann et al., 2008). In this study, we used RA to activate meiosis in primordial germ cells (PGCs) and further clarify *Piwil1* gene function during meiosis in poultry.

1.1. Animals and main experimental apparatus

All animals and eggs were purchased from the Langshan Chicken Breeder Farm in Nantong, Jiangsu province, China, including 10 male Langshan chickens (*Gallus gallus*) at 28 weeks of age and 280 hatching eggs. One hundred and eighty eggs were incubated for 4.5 days, and the other 100 eggs were incubated for 18 days in an incubator (Nanjing Wansheng Incubation Equipment Co., Ltd., Nanjing, China) at a temperature of 38 °C and a relative humidity of 60%. All experimental procedures were carried out in accordance with the rules of the Animal Experimental Committee of Yangzhou University.

1.2. Collection of different types of spermatogenic cells

1.2.1. Preparation and isolation of primordial germ cells (PGCs) and spermatogonial stem cells (SSCs)

We used 180 4.5-day-old Langshan chick embryos to get primordial germ cells (PGCs) and 100 18-day-old embryos to get spermatogonial stem cells (SSCs). The two cell types were separated as described by Li et al. (2002) and Sun et al. (2011). Both cell types were cultured in standard stem cell knock-out DMEM culture medium (Life Technologies, Shanghai, China) supplemented with 10% fetal calf serum (FBS) (Hyclone, South Logan, Utah, USA), 2% chicken serum (Life Technologies, New Zealand), 2 mmol/L L-glutamine (Gibco, Life Technologies, China), 1 mmol/L sodium pyruvate (Gibco, Life Technologies, China), 5.5×10^{-5} mol/L β-mercaptoethanol (BBI, Toronto, Ontario, Canada), 0.1 mmol/L non-essential amino acids (Gibco, Life Technologies, Shanghai, China), 5 ng/mL human stem cell growth factor (Sigma–Aldrich, China), 10 ng/mL mouse leukemia inhibitory factor (Sigma–Aldrich, China), 10 ng/mL fibroblast growth factor (Sigma–Aldrich, China), and 100 U/mL of a penicillin–streptomycin combination (Life Technologies, China). Cells were cultured in a CO_2 incubator (Thermo Fisher Scientific, China) at 37 °C and 5% CO_2. Some of the PGCs were used in the subsequent experiments, and some were used for RNA extraction. The SSCs were used for RNA extraction.

1.2.2. Chicken testis spermatogenic cell separation

1.2.2.1. Preparation of spermatogenic single cell suspension. We collected testes from two 28-week-old male Langshan chickens; removed the tunica serosa testis, albuginea, and auxiliary; washed them 3× with phosphate-buffered saline (PBS, 1X) (Hyclone, South Logan, Utah, USA); and used ophthalmic scissors cut them into pieces. The samples were then enzymatically digested by adding a 10-fold volume of collagenase IV (1 mg/mL) (Sigma–Aldrich, Shanghai, China) and shaking in a vapor-bathing constant temperature vibrator (Jintan Precision Instrument Factory, Changzhou, China) at 37 °C and 120 rpm for 20 min. Digestion was then stopped with 10% FBS, the samples were centrifuged for 10 min at 1000 rpm, and the supernatant was discarded. Treated with collagenase IV again. The samples were next digested with Trypsin 0.25% EDTA (Sigma–Aldrich, China), the second enzyme. A 5-fold volume of enzyme was added to the sample, which was then shaken in a vapor-bathing constant temperature vibrator at 37 °C and 120 rpm for 10 min. The digestion process was terminated with 10% FBS, the sample was centrifuged at 1000 rpm for 10 min, and the supernatant liquor was discarded. Digested sample withTrypsin 0.25% EDTA again and centrifuge with 1000 rpm/min, 10 min. The resulting pellet was resuspended in Dulbecco's Modified Eagle Media (DMEM) (Hyclone, Thermo Fisher Scientific, China) supplement 10% FBS, and then filtered through a 200-mesh screen that collected the filtrate. The resuspended sample was centrifuged at 1000 rpm for 5 min and then resuspended again in complete medium. This yielded our single-cell suspension.

1.2.2.2. Sorting different spermatogenic cells by flow cytometry. We prepared a propidium iodide (PI) (Sigma–Aldrich, Shanghai, China) solution using 1 mg PI powder diluted with 1 mL PBS to a concentration of 1 mg/mL. We then absorbed 500 μL 1 mg/mL PI solution into 500 uL Triton X-100 (Sigma–Aldrich, China) to obtain a 0.5 g/L PI working solution. The suspension was transferred into a 5-mL Eppendorf tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, then the cells were washed with PBS (1 ×). The cells were resuspended with 1 mL PBS (1 ×) added to 50 μL working solution. In order to mark