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Polycomb repressive complex 1 (PRC1) regulates meiotic initiation of ovarian germ cells in chick embryos

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ABSTRACT

Meiosis is essential for gametogenesis and exhibits sex-specific property during embryonic development. Retinoic acid (RA) signalling initiates germ cell meiosis by activating *Stra8* (stimulated by RA gene 8). Although additional factors are involved in regulating the meiotic initiation of germ cells, their regulatory mechanisms are unclear. In this study, we found that Polycomb repressive complex 1 (PRC1) is largely expressed in chicken ovarian germ and somatic cells during early stages of meiosis. We demonstrated that PRC1 regulates *Stra8*, pluripotent factors and paracrine factors (Notch ligands) leading to a synergistic effect on the suppression of germ cell meiotic initiation. Finally, we observed that repression of PRC1 resulted in precocious meiotic initiation and apoptosis of ovarian cells *in vivo*. These results aid in understanding the regulation of meiotic initiation in germ cells by PRC1 and provide evidence to support the hypothesis that regulation of meiotic initiation is conserved in higher vertebrates.

1. Introduction

Proliferation and differentiation are two main events that occur during the development of pluripotent stem cells. In primordial germ cells (PGCs), the proliferative process switches to the differentiation process at the initiation of meiosis, which is crucial for gametogenesis. In the mouse embryo, PGCs are first detected around embryonic day 6.25 (E6.25), and are observed to migrate into the gonadal ridges from E9.5 to E11.5 (Ginsburg et al., 1990). Subsequently, meiotic initiation of PGCs occurs and is determined by extrinsic environmental cues. In foetal ovaries, retinoic acid (RA) signalling initiates meiosis in PGCs by activating Stra8 (stimulated by RA gene 8) (Baltus et al., 2006; Koubova et al., 2006). RA is an active metabolite of vitamin A that is synthesized by retinaldehyde dehydrogenases (RALDHs) in the mesonephros and is transported by mesonephric tubules that are physically connected with the anterior end of the gonad (Menke et al., 2003). RA is also produced in the mesonephros of male embryos, but the P450 enzyme CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1) that is synthesized by somatic cells in the testis, degrades RA and prevents meiotic initiation (Bowles et al., 2006). Subsequently, the proliferation of PGCs in the testis is progressively slowed down and PGCs remain in the G0/G1 stage of mitosis until birth.

To explain the abrupt meiotic initiation in female germ cells at E13.5, *Stra8* and other genes of the early meiosis programs are hypothesized to be repressive until RA signalling reaches a certain threshold (Griswold et al., 2012). A recent report demonstrated that the establishment of this RA dosage threshold depends on the functions of Polycomb repressive complex 1 (PRC1), which directly suppresses the capability of *Stra8* to respond to RA signalling and maintains germ cell proliferation by regulating the expression of pluripotency genes (Yokobayashi et al., 2013). And an earlier report demonstrated that the deletion of PRC1 subunit, Cbx2, is exhibited premature meiosis onset in male mouse germ cells (Baumann and De La Fuente, 2011). PRC1 is a multi-subunit protein complex with a chromatin-modifying function. Five Polycomb group (PcG) protein families contribute to the subunit composition of PRC1 complexes, with each protein family comprised of several members, including Cbx2/4/6/7/8, Ring1A/B, PHC1/2/3, PCGF1/6, and RYBP/YAF2 (Di Croce and Helin, 2013). PRC1 complexes have diverse composition, but all PRC1 complexes contain the core components comprising of RNF2 and one of the PCGFs. These two subsets have a basal catalytic activity and then interact with additional subsets that regulate enzymatic activity or define the mode of recruitment

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to the targeting loci (Aranda et al., 2015). By catalysing mono-ubiquitination of histone H2A at lysine 119 (H2AK119ul), which leads to chromatin compaction and this compact state reduces the accessibility of transcription factors as well as ATP-dependent chromatin-remodelling machineries (Francis et al., 2004), PRC1 plays a key role in stem cell identity, self-renewal, and differentiation. Although the PcGs forming the PRC1 complex are conserved in many different species, whether PRC1 controls the time of meiotic entry in other species is unknown.

Compared to mammals, early chicken embryo development has some distinct processes. Firstly, mouse PGCs migrate within the gut epithelium and arrive at the gonadal ridges, whereas chicken PGCs migrate into the gonadal ridges via embryonic vasculature (Stern, 2005). Secondly, In male chicken embryos, both gonads develop into functional testes, but in the female only the left gonad develops into a functional ovary while the right gonadal tissue remains rudimentary (Levin et al., 1995). Interestingly, meiotic initiation in the chicken embryo is also decided by RA, and although RA is generated by RALDHs in both sexes, it is degraded by CYP26B1 in the testis (Smith et al., 2008; Yu et al., 2013). This process is similar to meiotic initiation in mice. Therefore, RA may have a conserved role in regulating meiotic initiation in higher vertebrates. However, this hypothesis needs more evidence to be proven.

The gonad is composed of several lineages of somatic cells as well as germ cells. Before meiosis is initiated in the germ cells, the cells that surround these germ cells are the Sertoli cells in the testis and the foetal granulosa cells in the ovary. In the mouse embryo, paracrine factors secreted by somatic cells regulate the meiotic initiation of germ cells. Wnt4/Rspo signalling regulates β-catenin to suppress germ cell proliferation and promote meiotic initiation (Chassot et al., 2011). FGF9 suppresses meiotic initiation by maintaining the pluripotency of germ cells and promoting the expression of genes related to the male sexual fate commitment (Bowles et al., 2010). FGF9 also contributes to the up-regulation of NANOS2, an RNA-binding protein that is critical for initiating the male pathway in germ cells, to suppress meiotic initiation (Bowles et al., 2010). Notch signalling promotes RA-dependent stimulation of *Stra8* by maintaining an activated epigenetic state of *Stra8* (Feng et al., 2014a). PRC1 regulates a number of signalling pathways related to cell proliferation and differentiation in mammalian stem cells and cancer cell lines. However, whether PRC1 affects meiotic initiation by controlling paracrine factors from ovarian somatic cells is unknown.

In this study, we used ovarian tissue and cell culture, together with an *in vivo* approach to examine the function and mechanism of PRC1 in meiotic initiation of chicken germ cells. We demonstrated that PRC1 directly repressed *Stra8* and indirectly maintained the expression of pluripotent genes to protect chicken germ cells from premature shifting of proliferation to meiosis. In addition, we identified that PRC1 repressed paracrine factors and Notch ligands, resulting in a synergistic effect on meiotic initiation.

2. Materials and methods

2.1. In vitro culture of embryonic ovarian tissues

Fertilized Hyline chicken (*Gallus gallus*) eggs were incubated at 38 °C and 60% humidity. Individual left ovaries without the mesonephros at E12.5 were placed separately on 12-well culture plates (Costar, Corning, NY) with 1 ml of serum-free ITS culture medium: Dulbecco minimum essential medium (DMEM, Gibco, Carlsbad, CA) containing 10 μg/ml insulin, 5 μg/ml transferrin and 3×10^{-8} M sodium selenium supplement. According to different experiments, the cultured ovarian tissues were treated with RA (1 μM, Sigma-Aldrich, St. Louis, MO), PRT4165 (50 μM, Merck Millipore, Bedford, MA), or WIN18446 (1 μM, Tocris Bioscience, Bristol, UK), or LE540 (2 μM, Wako, Japan). These chemicals were dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich) such that the final concentration of DMSO in the medium was less than 0.1%.

2.2. Injection of embryos in vivo

RA and PRT4165 were dissolved in DMSO to a concentration of 1 mM and 50 mM, respectively. Eggs were windowed under sterile conditions and injections were applied to the air sac at E12.5. Each egg was injected with 50 μl DMSO containing RA or PRT4165. The control embryo received 50 μl DMSO. After injection, the windows were sealed with paraffin and the eggs were incubated in an egg incubator until the left ovaries of the embryos were collected to perform the immunofluorescence and TUNEL assay.

2.3. Immunofluorescence detection

The ovaries were fixed in 4% paraformaldehyde and embedded in Tissue-Tek OCT compound (Sakura Finetek, Japan). Cryosections were cut at a thickness of 10 μm to perform immunostaining. The cryosections were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 20 min, then permeabilised with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. After incubation with the blocking buffer containing 5% bovine serum albumin (BSA, Solarbio, Beijing, China) for 20 min, the cells were stained overnight with the SYCP3 antibody (1:500 dilution, Novus Biologicals, Littleton, CO) at 4 °C and rewarmed at 37 °C for 45 min. Then, the Alexa Fluor 594 goat anti-rabbit IgG (1:1000, Abcam, Cambridge, UK) was added and incubated at room temperature for 1 h. Nuclei were stained with 10 μM DAPI (Sigma-Aldrich) for 30 min. Fluorescence images were obtained using the fluorescence microscope, IX71 (OLYMPUS, Tokyo, Japan).

2.4. Immunohistochemistry and TUNEL assay

Ovaries were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were obtained by cutting the samples at a thickness of 5 μm. The sections were treated with citrate buffer (0.01 M, pH 6.0) and then heated in a microwave oven for 10 min for antigen retrieval. The sections were then digested with proteinase K (20 μg/ml) for 15 min at room temperature. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 30 min at room temperature. For immunohistochemistry, the sections were blocked with 10% normal goat serum (Solarbio) at 37 °C for 30 min, and the sections were then incubated overnight with the primary antibody against Rnf2 (1:100, Abcam) at 4 °C. The sections were then incubated with biotinylated goat anti-rabbit IgG (Abcam) for 30 min at room temperature. After incubating with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, staining was developed with anti-rabbit HRP/DAB detection kit (Abcam). Control sections were treated with normal goat serum instead of the primary antibody. For the TUNEL assay, the sections were stained using an *in situ* apoptosis detection kit (Promega, Madison, WI, USA) according to the manufacturer's protocols with some modifications. Following the H_2O_2 treatment step, the end-labelling reaction was performed by adding the rTdT reaction mix for 1 h at 37 °C. The rTdT enzyme reaction was terminated with the stop buffer for 15 min at room temperature. The streptavidin-HRP solution was added onto the sections and incubated for 30 min at room temperature. DAB solution was then added and was incubated for 10 min at room temperature. The sections for immunohistochemistry and TUNEL assay were both stained with haematoxylin and were observed with a light microscope, PX-50 (OLYMPUS, Tokyo, Japan).