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Chd9 mediates highly loosened chromatin structure in growing mouse oocytes

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ABSTRACT

During oogenesis, oocytes prepare for embryonic development following fertilization. The mechanisms underlying this process are still unknown. Recently, it has been suggested that a loosened chromatin structure is involved in pluripotency and totipotency in embryonic stem (ES) cells and early preimplantation embryos, respectively. Here, we explored chromatin looseness in oocytes by fluorescence recovery after photobleaching (FRAP) using enhanced green fluorescent protein-tagged histone H2B. The results indicated that the chromatin in growing oocytes was already highly loosened to a level comparable to that in early preimplantation embryos. To elucidate the mechanism underlying the loosened chromatin structure in oocytes, we focused on chromodomain helicase DNA binding protein 9 (Chd9), which is highly expressed in growing oocytes. The oocytes from *Chd9* knockout mice (*Chd9*$^{-/-}$) generated using the CRISPR/Cas9 system exhibited a less loosened chromatin structure than that of wild-type mice, suggesting that *Chd9* is involved in the loosened chromatin structure in growing oocytes. These results suggest that a loosened chromatin structure, which is mediated by Chd9, is a prerequisite for the acquisition of totipotency after fertilization.

1. Introduction

Oogenesis is considered the process during which oocytes acquire the capabilities required for ontogenesis. During this process, oocytes are likely to prepare for the acquisition of totipotency after fertilization. However, the mechanism governing this process has not been elucidated.

One hypothesis is that there is a correlation between loosened chromatin structure and the plasticity or differentiation potential of embryos and embryonic stem (ES) cells. A previous study revealed that ES cells had less heterochromatin-rich and more euchromatin-rich regions than differentiated somatic cells, and that this chromatin characteristic disappeared upon the induction of differentiation [1,2]. In the chromatin with a loosened structure in ES cells, histones are exchanged at a higher rate compared to those in differentiated somatic cells. The mobility of histone proteins can be analyzed by fluorescence recovery after photobleaching (FRAP) using fluorescent protein-fused histone proteins. Our previous study analyzing chromatin structure by FRAP using enhanced green fluorescent protein-fused histone 2B (eGFP-H2B) revealed that the mobility of eGFP-H2B was much higher in zygotes than in ES cells, and histone mobility was higher in ES cells than in differentiated cells [3], indicating that histone mobility is extremely high in zygotes. In addition, the mobility of eGFP-H2B decreased with the progression of preimplantation development, during which plasticity is gradually lost [3,4]. In colonies of ES cells, a fraction of the cells exhibit higher differential potential than others. These cells contributed to the production of extra-embryonic tissues as well as embryos, and they have high histone protein mobility at levels comparable to those of 2-cell stage embryos [5,6]. Thus, the mobility of histone proteins seems to be important for plasticity and differentiation potential.

There is some evidence suggesting that chromatin structure is loosened in oocytes, even though they are terminally differentiated. Generally, the expression of repetitive sequences is silenced to maintain genomic integrity in differentiated cells. However, retroelements are transcriptionally active and utilized as regulatory components for gene expression in preimplantation embryos and ES cells [5,7], which seems to be caused by the loosened chromatin

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structure of these cells. Interestingly, the transcription of retroelements and their involvement in the regulation of gene expression are also observed in oocytes [7], suggesting that oocytes have a loosened chromatin structure. Furthermore, oocyte chromatin contains the histone variants that cause loosened chromatin structure. Linker histones play an important role in the regulation of chromatin structure. Oocyte-specific linker histone (H1foo) is expressed during oogenesis [8]. When H1foo is expressed in differentiated fibroblasts, the heterochromatin regions are decreased, suggesting that H1foo is involved in the establishment of loosened chromatin structure [9]. The histone H3 composition in oocytes is similar to that in zygotes. There are three types of H3 variants, H3.1, H3.2, and H3.3. H3.3 is dominant in oocytes as well as in zygotes [10,11]. When CAF1, which deposits H3.1 and H3.2 into chromatin to establish heterochromatin, is knocked-down in ES cells (and H3.3 is deposited into chromatin instead of H3.1 and 3.2), histone mobility is increased [12], suggesting that H3.3 deposition increases histone mobility. Thus, the chromatin structure of oocytes in which H1foo and H3.3 are enriched seems to be loosened. However, there is no direct evidence that oocytes have loosened chromatin structure, and the molecular mechanism regulating chromatin looseness in oocytes remains unknown.

The Chd family comprises chromodomain helicase DNA binding proteins that alter chromatin structure [13,14]. Our previous RNA-seq analysis revealed that *Chd9* is one of the Chd family members expressed at high levels in oocytes [15]. The analysis of the genome-wide distribution of Chd family members in ES cells by chromatin immunoprecipitation-sequencing (ChIP-seq) reported that the distribution of Chd9 correlated with DNase-sensitive promoters, which are a hallmark of loosened chromatin structure, suggesting that Chd9 is involved in the regulation of loosened chromatin structure [16].

In the present study, we showed that the chromatin structure in growing oocytes is loosened, and that Chd9 is involved in the mechanism regulating this loosened chromatin structure.

2. Materials and methods

2.1. Collection and culture of oocytes and embryos

Growing oocytes were obtained from 12-day-old BDF1 female mice (SLC, Inc., Shizuoka, Japan). They were subjected to microinjection for FRAP analysis. Preimplantation embryos were prepared by *in vitro* fertilization. The unfertilized oocytes were obtained from 20 to 24-day-old BDF1 female mice, which had been injected with 5 IU of equine chorionic gonadotropin (eCG; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and human chorionic gonadotropin (hCG; ASKA Pharmaceutical Co., Ltd.) at 46–50-h intervals. Sixteen hours after hCG injection, the unfertilized oocytes were collected and then inseminated with capacitated spermatozoa that had been collected from mature ICR male mice and cultured in human tubal fluid (HTF) medium supplemented with 10 mg/mL bovine serum albumin (BSA) for 2 h in a humidified atmosphere of 5% CO_2 at 38 °C. Two hours after insemination, the fertilized oocytes were washed with KSOM medium and then subjected to microinjection for FRAP analysis.

All procedures using animals were reviewed and approved by the University of Tokyo Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

2.2. FRAP analysis

The experiments were performed essentially as described in Ooga et al. [3], with slight modifications. Complementary RNA (cRNA) encoding eGFP-H2B was injected into the oocytes and embryos at a concentration of 500 ng/μL. A total of 12 photographs were taken at 5-s intervals. The mobile fraction (MF), which is an index of histone mobility, was calculated using the following equation:

$$MF = (F_{end} - F_{post})/(F_{pre} - F_{post}),$$

where F_{end} is the relative intensity of fluorescence at the endpoint, F_{post} is soon after photo-bleaching, and F_{pre} is before photo-bleaching.

2.3. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Organs (brain, lungs, heart, liver, kidneys, and ovaries) were obtained from 8-week-old female BDF1 mice, and testes were obtained from males. Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and 1 μg of RNA was reverse transcribed using oligo dT primers. The obtained cDNA was used for PCR with Ex Taq polymerase. The primers used were as follows: *Chd9*, 5′-GGGAT CTTATCACCCCCACT-3′ (forward) and 5′-CAGCATCCTTACACGAAG CA-3′ (reverse); and *Gapdh*, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). The PCR conditions were as follows: 95 °C for 2 min followed by 27 (*Chd9*) and 25 (*Gapdh*) cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The amplified DNA fragments were analyzed on 2% agarose gel, and images were obtained using a DT-20MP UV illuminator (Atto Corporation, Tokyo, Japan).

2.4. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from 15 oocytes and preimplantation embryos with ISOGEN and used for cDNA synthesis. Random hexamers were used as primers for reverse transcription. The obtained cDNA was used for PCR with SYBR Premix Ex Taq II (TaKaRa Bio Inc., Tokyo, Japan). qPCR reactions using the primer set for *Chd9* shown above were performed using a Thermal Cycler Dice Real-Time System (TaKaRa Bio, Inc.) following manufacturer's instructions. Rabbit alpha-globin was used as an external control, and the primer set used was as follows: 5′-GTGGGACAGGAGCTTGAAAT′ (forward) and 5′- GCAGCCACGGTGGCGAGTAT-3′ (reverse).

2.5. Construction of single guide RNA (sgRNA) coding vector

The target sequence for *Chd9* on sgRNA was 5′-TCTGGGAAGTT-CATGTGCAC-3′. To construct an expression vector with a T3 promoter driving sgRNA expression, the primers 5′- GAAATTAACCC TCACTAAAGGtctgggaagttcatgtgcacGTTTTAGAGCTAGAAATAGC-3′ (forward, lower case letters indicate the sequence of the region targeting *Chd9*) and 5′-TTTAAAAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAA AAC-3′ (reverse) were used, as described previously [17]. To construct an sgRNA expression cassette, these primers were used in PCR amplification without any template DNA, using Ex Taq DNA polymerase (TaKaRa Bio Inc.). The PCR conditions were as follows: 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. As a result, the sgRNA expression cassette was obtained as a primer dimer. This PCR product was cloned into a pCRII TOPO vector (Life Technologies, Carlsbad, CA, USA) and then the sequence was confirmed. The resulting plasmid was named pTOPO-sgRNA-Chd9.