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—Original Article—

Oocyte-specific gene *Oog1* suppresses the expression of spermatogenesis-specific genes in oocytes

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Abstract. *Oog1*, an oocyte-specific gene that encodes a protein of 425 amino acids, is present in five copies on mouse chromosomes 4 and 12. In mouse oocytes, *Oog1* mRNA expression begins at embryonic day 15.5 and almost disappears by the late two-cell stage. Meanwhile, OOG1 protein is detectable in oocytes in ovarian cysts and disappears by the four-cell stage; the protein is transported to the nucleus in late one-cell to early two-cell stage embryos. In this study, we examined the role of *Oog1* during oogenesis in mice. *Oog1* RNAi-transgenic mice were generated by expressing double-stranded hairpin *Oog1* RNA, which is processed into siRNAs targeting *Oog1* mRNA. Quantitative RT-PCR revealed that the amount of *Oog1* mRNA was dramatically reduced in oocytes obtained from *Oog1*-knockdown mice, whereas the abundance of spermatogenesis-associated transcripts (*Klhl10*, *Tekt2*, *Tdrd6,* and *Tnp2*) was increased in *Oog1* knockdown ovaries. *Tdrd6* is involved in the formation of the chromatoid body, *Tnp2* contributes to the formation of sperm heads, *Tekt2* is required for the formation of ciliary and flagellar microtubules, and *Klhl10* plays a key role in the elongated sperm differentiation. These results indicate that *Oog1* down-regulates the expression of spermatogenesis-associated genes in female germ cells, allowing them to develop normally into oocytes.

Key words: Mouse, Oogenesis, *Oog1*, RNAi, Spermatogenesis

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Oog1 is an oocyte-specific gene that is expressed at embryonic day (E) 15.5 when oocytes are progressing through meiotic prophase I; the mRNA persists through the two-cell stage [1]. The OOG1 protein contains a leucine zipper structure that contributes to its transcriptional activity [2]. OOG1 belongs to the leucine-rich repeat (LRR) superfamily [1]; LRR is a motif involved in protein-protein interactions [3]. Previously, in a yeast two-hybrid screen of a germinal vesicle (GV) oocyte cDNA library, we showed that OOG1 interacts with Ras and Ral guanine nucleotide dissociation stimulator (RalGDS); however, the function of OOG1 remains unknown. A GFP reporter assay revealed that the 3.9-kb *Oog1* promoter region is responsible for dimorphic expression in male and female germ cells and that *Oog1* expression begins at the pachytene stage [4].

During oogenesis, it is necessary to maintain a proper balance in the expression of sexually dimorphic genes. During gametogenesis, this balance is mediated by oocyte-specific transcription factors such as *Figla* and *Nobox*. Knockout of *Figla* in female mice increases the expression of spermatogenesis-associated genes in oocytes and inhibits the formation of primordial follicles [5]. In ovaries lacking *Nobox,* an oocyte-specific homeobox gene, the expression of spermatogenesis-associated genes such as *Dmrt1* and *Tekt2* is significantly upregulated, whereas that of oocyte-specific genes such as *Oog1* and *H1foo* is drastically downregulated [6, 7]. Although several genes related to oogenesis, such as *Figla* and *Nobox,* have been analyzed by knockout approaches, it is difficult to completely knock out *Oog1* because it is present in five copies on chromosomes 4 and 12.

To address this issue, we used an RNA interference (RNAi) approach [8, 9]. In this system, the *Oog1*-knockdown (KD) mice express *Oog1* double-stranded RNA (dsRNA) under the control of the 3.9-kb *Oog1* promoter to induce RNAi targeting *Oog1* only in cells where *Oog1* is endogenously expressed. In a previous study, the 3.9-kb *Oog1* promoter was shown to have strong activity starting in the pachytene stage of oogenesis [3]. We found that RNAi effectively and specifically decreased *Oog1* expression levels in mouse oocytes. Moreover, in *Oog1*-KD oocytes, spermatogenesis-associated gene transcripts (*Klhl10*, *Tekt2*, *Tdrd6,* and *Tnp2*) were upregulated in comparison to oocytes from wild-type (WT) mice. These results suggest that *Oog1* plays important roles in sex-specific differentiation, and controls oogenesis by inhibiting the expression of spermatogenesis-associated genes in the ovary.

Materials and Methods

Generation of transgenic mice

To generate transgenic (TG) mice expressing a long dsRNA target-

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Fig. 1. Structure of the *Oog1*-RNAi transgene. The *Oog1* inverted repeat transcript was expressed under the control of the 3.9-kb *Oog1* promoter. The expressed *Oog1* transcripts were down-regulated in transgenic mouse oocytes.

ing *Oog1*, an *Oog1* inverted repeat (IR) sequence was constructed downstream of AcGFP1-mem (Clontech Laboratories, Mountain View, CA, USA). The transgene was designed to be controlled by the 3.9-kb *Oog1* promoter (Oog1pro3.9-AcGFP-Oog1IR) (Fig. 1) [3]. The *Oog1* IR sequence contains sense and antisense target sequences 492 to 1193 bp after the transcription start site. When the *Oog1* IR sequence is transcribed, siRNAs are generated by Dicer function. The resultant siRNAs are recruited to the RNA-induced silencing complex, which induces degradation of complementary mRNA [10].

Linearized transgene fragments were purified and microinjected into the male pronucleus of one-cell embryos obtained from C57BL/6J mice (CLEA Japan, Tokyo, Japan). Microinjected embryos that reached the two-cell stage were transferred into oviducts of pseudopregnant ICR female mice (Japan SLC, Hamamatsu, Japan). TG mice were identified by PCR genotyping using the following AcGFP primer pair: sense, 5′-TGTTCACCGGCATCGTGCCC-3′; antisense, 5′-CTCGGCCGCGACTTGTAGT-3′ that produces a 314 bp product. Five TG founders were obtained: two Oog1pro3.9-AcGFP-Oog1IR1 males (lines A and B) and three Oog1pro3.9-AcGFP-Oog1IR1 females (lines C, D, and E). Female offspring obtained from crossing TG males (line A or B) with WT females were used for the studies described here. Although both TG lines exhibit almost the same phenotype, line B was used for gene expression analyses because it harbored more copies of the transgene (22 copies, data not shown).

Embryo and oocyte collection

Four- to twelve-week-old C57BL/6J TG and WT female mice (Japan SLC) were superovulated by injection of 5 IU of equine chorionic gonadotropin (eCG; ASKA Pharmaceutical, Japan) followed by 5 IU of human chorionic gonadotropin (hCG; ASKA) 48 h later.

Embryos were collected from C57BL/6J WT female mice after mating with C57BL/6J WT males. At 18 h after hCG injection, all embryos were flushed from oviducts in potassium simplex optimization medium (KSOM) supplemented with amino acids [11] and 4 mg/ml BSA. After flushing, embryos were cultured to the following stages in KSOM: early one-cell (18 h after hCG injection), late one-cell (30 h), early two-cell (32 h), late two-cell (48 h), and four-cell (54 h). GV and metaphase II (MII) oocytes were collected from C57BL/6J TG and WT female ovaries. GV oocytes were collected by puncturing mouse ovarian follicles at 44–48 h after eCG injection with 26-gauge needles. Unfertilized MII oocytes were collected at 16 h post-hCG from oviductal ampullae. Both GV and MII oocytes were treated with 1% hyaluronidase to remove cumulus cells.

Histology and immunohistochemistry

Ovaries from TG and WT female mice at postnatal day (PND) 1, 7, or 19, or at 6 months of age, were fixed by incubation in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Paraffin-embedded ovaries were cut into 5 μm-thick sections. For histology, sections were stained with Carrazzi's Hematoxylin Solution (Wako Pure Chemical Industries, Osaka, Japan), counterstained with Eosin Y solution (Millipore–Sigma, St. Louis, MO, USA), and imaged by light microscopy. For immunohistochemistry, sections were incubated in blocking buffer (1% goat serum, 0.01% Tween-20 in PBS) for 2 h at room temperature or overnight at 4°C, and then stained with rabbit anti-OOG1 antibody (1:50 dilution; Oog1-2001) in blocking buffer [1]. After washing with PBS, embryos were incubated in PBS-T containing secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:200 dilution, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature (24°C). After three washes in PBS and two immersions in PBS for 5 min each, nuclei were stained with Hoechst 33342 (1:1000 dilution; Millipore–Sigma) in blocking buffer. Sections were rinsed three times in PBS and mounted with 50% glycerol in PBS. Fluorescent signals were detected using a fluorescence microscope (BX50, Olympus, Tokyo, Japan).

RNA extraction and quantitative RT-PCR

RNA extraction and quantitative RT-PCR (qPCR) were performed as previously described [12]. Total RNAs from 20 oocytes and embryos at each developmental stage were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). After treatment with RNase-free DNase I (Roche, Basel, Switzerland), reverse transcription was performed on isolated RNA using ReverTra Ace (Toyobo, Osaka, Japan) with random primers (Invitrogen). Transcript levels were determined on a Rotor-Gene 6000 (Qiagen, Venlo, Netherlands) and normalized against the corresponding levels of *Gapdh*; relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [13]. The following primers were used for qPCR: *Oog1*: sense, 5′- AGTGTTACCCTGCCCCTCTA -3′; antisense, 5′- TCCAGAAGCTCAGGACAAAAA -3′ (88 bp product); *Gapdh* (internal control): sense, 5′- CGTGTTCCTACCCCCAATGT -3′; antisense, 5′- TGTCATCATACTTGGCAGGTTTC -3′ (73 bp product); *Adad1*: sense, 5′- TAGAACCAGCAGGACCACCT -3′; antisense, 5′- ACTGGACTTGTCTCCCTTCA -3′ (99 bp product); *Crisp2*: sense, 5′- GCATGCCCTCCTGATCCTAA -3′; antisense, 5′- AAGCCATGGCCGAAAGTGTT -3′ (89 bp product); *Dkkl1*: sense, 5′- GTGTCGACTGAGGGTCTTGC -3′; antisense, 5′- AGGTGTTCTGCTGAGAGTCG -3′ (95 bp product); *Klhl10*: sense, 5′- AGTGGCCCCTATGCACTCTA -3′; antisense, 5′- AGACGCACGTAGCCATCAAA -3′ (96 bp product); *Spata19*: sense, 5′- TGTCCAAAGAGTGTCCACCTCA -3′; antisense, 5′- CAAGGGCTCAGCGTTTAGAGT -3′ (81 bp product); *Tekt2*: sense, 5′- ACATGACAATAGGACCCGCC -3′; antisense, 5′- CACTTGTCCAGCGTCTCCTT -3′ (72 bp product); *Tdrd6*: sense, 5′- TTGCGTGTCTACTGAAATAGGTG -3′; antisense, 5′-

ACTTTATTCAAATGTTGGGTTGC -3′ (81 bp product); *Tnp2*: sense, 5′- GTAGCTCAGGGCGAAGATACAA -3′; antisense, 5′- TTCCTGTGACATCATCCCAAC -3′ (110 bp product).

Statistical analyses

Each experiment was repeated at least twice. All data are expressed as the mean ± s.e.m. Statistical analysis was performed by Student's *t*-test. P-values < 0.05 were considered to be statistically significant.

Ethical approval for the use of animals

All animal experiments were approved by the Animal Research Committee of Kyoto University (Permit Numbers: 28–17 and 29–17) and were performed in accordance with the committee's guidelines.

Results

Expression of Oog1 mRNA

Analysis of *Oog1* transcript levels in oocytes and embryos by qPCR revealed that *Oog1* was highly expressed in GV and MII oocytes, with the highest expression at the GV stage (Fig. 2). Moreover, *Oog1* expression gradually decreased as the embryos developed, and was almost eliminated from embryos at the four-cell stage. These data are consistent with previous reports that *Oog1* mRNA disappears after the two-cell stage [1]. Considering that zygotic genome activation occurs at the two-cell stage in mouse embryos, these results suggest that *Oog1* is a maternally expressed gene.

Inhibition of the Oog1 gene and OOG1 protein

To investigate the knockdown efficiency of *Oog1* in *Oog1*-KD mice, we performed qPCR to measure the abundance of *Oog1* transcripts in GV oocytes from WT and *Oog1*-KD mice. *Oog1* mRNA expression was significantly diminished in *Oog1*-KD mice (Fig. 3A); specifically, the amount of *Oog1* mRNA was reduced by 90% in GV oocytes from *Oog1*-KD mice. Furthermore, to confirm suppression of the OOG1 protein, we performed immunofluorescence staining in *Oog1*-KD and WT mice at PND 7 and 19. OOG1 signals were detected in oocytes from WT mouse ovaries, but not in those from *Oog1*-KD mouse ovaries (Fig. 3B).

Histological phenotypes of Oog1-KD mice

To compare histological phenotypes between *Oog1*-KD and WT mice, we collected the ovaries of PND 1, 7, and 19 and 6-month-old mice to perform hematoxylin-eosin (HE) staining. Based on the histological analysis, the numbers of oocytes, primordial follicles, primary follicles, and secondary follicles did not significantly differ between *Oog1*-KD and WT ovaries until PND 19 (data not shown). However, in 6-month-old mice, we detected a few abnormal oocytes in the ovaries of *Oog1*-KD mice compared to the WT ovaries (Fig. 4). Eight-week-old *Oog1*-KD mice generated offspring normally, and litter sizes did not significantly differ between *Oog1*-KD and WT mice (data not shown).

Expression of spermatogenesis-associated genes in Oog1-KD ovaries

Previous work showed that some spermatogenesis-associated genes are overexpressed in *Figla*-null mouse oocytes but suppressed in the

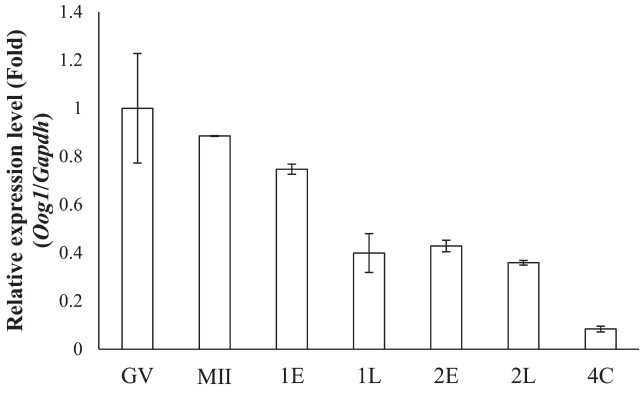

Fig. 2. Quantitative PCR of oocytes and embryos at each developmental stage. Expression ratios were obtained by dividing the relative amount of *Oog1* (*Oog1*/*Gapdh*) at each stage. The mRNA levels of the germinal vesicle (GV) oocytes were defined as 1. The expression of *Oog1* gradually decreased as oocytes developed and was almost eliminated in embryos at the four-cell stage (n = 2). GV: GV stage, MII: metaphase II stage, 1E: early one-cell stage, 1L: late one-cell stage, 2E: early two-cell stage, 2L: late two-cell stage, 4C: four-cell stage.

TSPY1-Figla TG mouse, in which *Figla* is ectopically expressed in male germ cells [14]. Hence, we investigated whether OOG1 affects the expression of spermatogenesis-associated genes. To this end, we performed qPCR to measure the expression of *Adad1, Crisp2*, *Dkkl1*, *Klhl10*, *Spata19*, *Tekt2*, *Tdrd6,* and *Tnp2* transcripts in WT and *Oog1*-KD ovaries. *Klhl10*, *Tekt2*, *Tdrd6,* and *Tnp2* mRNA were significantly upregulated in *Oog1*-KD mice (Fig. 5), whereas the expression of *Adad1, Crisp2*, *Dkkl1,* and *Spata19* did not differ significantly between *Oog1*-KD and WT mice. These results raised the possibility that during oogenesis, OOG1 participates directly or indirectly in the repression of genes that are normally expressed during spermatogenesis.

Discussion

Oog1 was highly expressed in GV and MII oocytes with the highest expression at the GV stage. This indicates that *Oog1* is transcribed actively at the GV stage, but the amount of *Oog1* mRNA gradually decreases after MII. Based on the qPCR and immunofluorescence results, *Oog1* mRNA and OOG1 protein were efficiently suppressed in *Oog1*-KD mice. Histologically, *Oog1*-KD female mice exhibit normal folliculogenesis, in contrast to *Figla*- or *Nobox*-deficient female mice, which have atrophic ovaries and cannot undergo follicle formation. Some oocyte-specific genes (so-called "maternal effect genes"), such as *Mater*, are required to initiate and complete embryogenesis; however, because *Oog1*-KD mice generate offspring normally, *Oog1* is not a maternal effect gene. Nonetheless, two of the previous studies showed that conditional knockout of *Setd1b* or *Fzr1* in mice resulted in ovarian failure after 8 weeks of age [15, 16]. The fact that *Oog1*-KD mice have abnormal oocytes at almost the same age as *Setd1b* or *Fzr1* conditional knockout mice suggests the possibility of a reproductive defect in older *Oog1*-KD mice. Further investigation using *Oog1*-KD mice older than 6 months

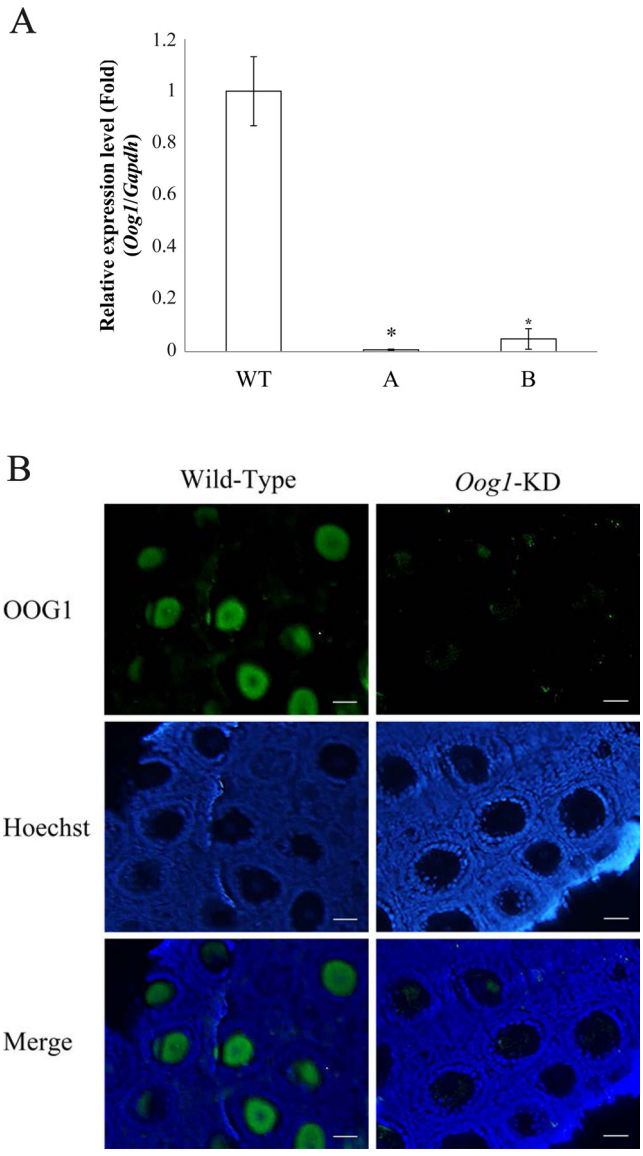

Fig. 3. *Oog1*-knockdown effect on levels of *Oog1* mRNA and protein in ovaries. (A) The suppression efficiency of *Oog1* in *Oog1*-knockdown (KD) mice. *Oog1* mRNA level was significantly reduced in *Oog1*-KD mice (*Oog1*pro3.9-Oog1 IR1, Line A and B). This figure shows that *Oog1* mRNA was significantly suppressed in *Oog1*-KD mouse GV oocytes (n = 3, * $P < 0.05$, *t*-test). (B) Immunofluorescence staining in WT and *Oog1*-KD mouse ovaries at postnatal day 19. OOG1 signals were detected in oocytes from WT, but not TG, mouse ovaries (Blue: Hoechst, Green: OOG1). Scale bars, 100 µm.

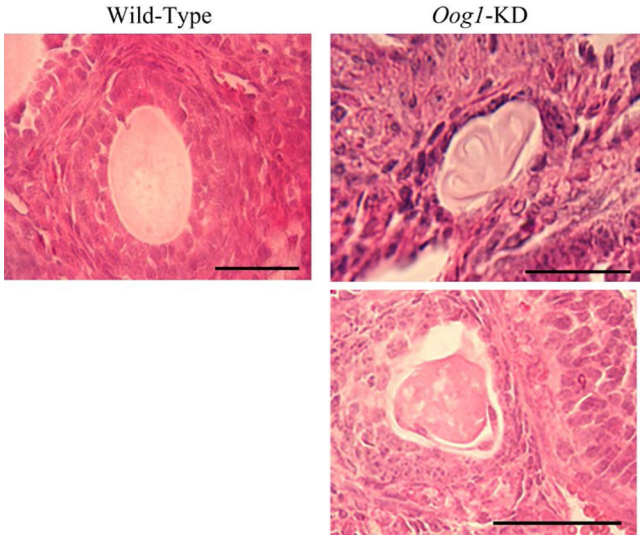

Fig. 4. Histology in *Oog1*-KD mouse ovaries. Hematoxylin-eosin staining in WT and *Oog1*-KD mouse ovaries at 6 months of age. Abnormal oocytes were observed in *Oog1*-KD mouse ovaries. Scale bars, 100 µm.

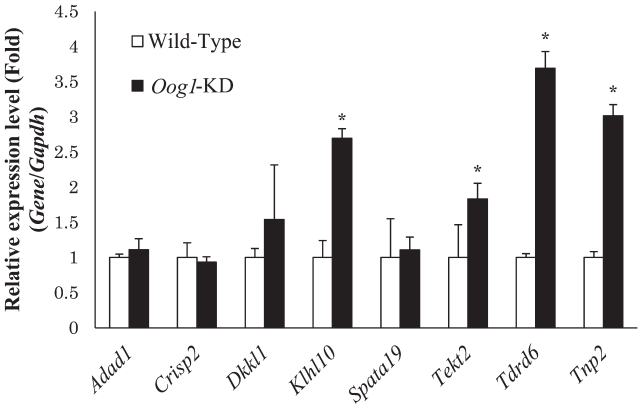

Fig. 5. Quantitative PCR of spermatogenesis-associated genes in ovaries. The expression of *Adad1, Crisp2*, *Dkkl1*, *Klhl10*, *Spata19*, *Tekt2*, *Tdrd6,* and *Tnp2* transcripts in WT and TG ovaries were quantitated by quantitative PCR. *Khl10*, *Tekt2*, *Tdrd6,* and *Tnp2* mRNAs were significantly upregulated in *Oog1*-KD mouse ovaries, whereas *Adad1, Crisp2*, *Dkkl1,* and *Spata19* were not significantly affected (n = 2, * $P < 0.05$, *t*-test).

will be required to determine whether *Oog1* knockdown causes an abnormality in oogenesis.

The expression of some spermatogenesis-associated genes was increased in *Oog1*-KD mouse ovaries, suggesting that *Oog1* is involved in the suppression of spermatogenesis-associated genes in ovaries. *Oog1* expression in germ cells starts at E15.5, corresponding to the pachytene stage in female germ cells. *Khl10*, *Tekt2*, *Tdrd6,* and *Tnp2*, whose expression was increased in *Oog1*-KD mouse ovaries, are expressed after the pachytene stage during spermatogenesis [17–20]. In particular, the expression of *Tdrd6* starts from the mid-pachytene stage and peaks after the late pachytene stage [19]. *Tdrd6* is essential for the formation of the chromatoid body (CB) that exists in male germ cells [19, 21]. In mice, the CB appears for the first time in the cytoplasm of meiotic pachytene spermatocytes and seems to coordinate the regulation of microRNA and RNA-decay pathways [22]. Judging from this result, and the fact that the CB is not present in female germ cells, *Oog1* may regulate genes related to the formation

of the CB in these cells. Previous work showed that expression of *Tdrd1* and *Tdrd6* is increased in the ovaries of *Figla*-null mice, but decreased in the testes of *Figla*-overexpressing mice; furthermore, the germ cells of *Figla*-overexpressing male mice do not have CBs [14]. These findings show that male mice overexpressing *Oog1* may have the same phenotype as *Figla*-overexpressing mice.

TNP2 protein is diffusely distributed over the anterior tip of the nuclei in step 10 spermatids and remains localized over the more anterior portion of the nucleus even in step 13 spermatids. In *Tnp2*-deficient mice, sperm heads become severely deformed, coincident with malformation of the acrosome [20]. Previously, we showed that the 3.9-kb *Oog1* promoter is highly active during the formation of the acrosome [3], suggesting that OOG1 prevents the expression of genes related to acrosome formation.

TEKT2 protein is present from the elongating spermatid stage to mature sperm [18]. TEKT2 is a component of ciliary and flagellar microtubules, and *Tekt2* KO mice are completely infertile [23]. Microarray analysis of *Nobox*-deficient newborn mice revealed elevation of *Tekt2* expression, similar to our result in *Oog1*-KD. *Tekt2* is also required for central spindle microtubule organization. *Klhl10* is expressed in step 1–16 spermatids, but the encoded protein is detected only in step 9–16. In addition, reduced levels of KLHL10 may disrupt the integrity of the intercellular bridges among spermatids [17]. We also performed qPCR on female-specific genes and found that expression of *H1foo*, a mouse oocyte-specific linker histone, was decreased by 80% in *Oog1*-KD mice (data not shown). Therefore, *Oog1* may downregulate the expression of spermatogenesis-associated genes allowing the normal differentiation of female germ cells into oocytes.

In previous research, *Rasd1*, which is a member of Ras superfamily of small GTPases, was shown to be necessary for the MI-MII oocyte transition [24]. *Rasd1* is also suggested to regulate cytokinesis and spindle formation. *Oog1* is known to interact with Ras and RalGDS [2], suggesting that *Oog1*-KD may induce age-dependent abnormal oocyte formation via the Ras signaling pathway.

In *Figla* knockout mice, spermatogenesis-associated genes are overexpressed, which disrupts normal oogenesis [5]. Based on the results of this study, we propose that OOG1 protein directly or indirectly suppresses spermatogenesis-associated gene expression, which leads to abnormal oocyte formation in older age mice. To confirm this hypothesis, it is necessary to perform chromatin immunoprecipitation analysis to identify the loci where OOG1 binds to promote or suppress the expression of related genes.

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