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Oocyte-specific gene knockdown by intronic artificial microRNAs driven by *Zp3* transcription in mice

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Abstract. Conditional knockout technology is a powerful tool for investigating the spatiotemporal functions of target genes. However, generation of conditional knockout mice involves complicated breeding programs and considerable time. A recent study has shown that artificially designed microRNAs (amiRNAs), inserted into an intron of the constitutively expressed gene, induce knockdown of the targeted gene in mice, thus creating a simpler method to analyze the functions of target genes in oocytes. Here, to establish an oocyte-specific knockdown system, amiRNA sequences against enhanced green fluorescent protein (EGFP) were knocked into the intronic sites of the *Zp3* gene. Knock-in mice were then bred with EGFP transgenic mice. Our results showed that *Zp3*-derived amiRNA successfully reduced EGFP fluorescence in the oocytes in a size-dependent manner. Importantly, knockdown of EGFP did not occur in somatic cells. Thus, we present our knockdown system as a tool for screening gene functions in mouse oocytes.

Key words: Artificial microRNA, Genome editing, Oocyte, Transgenic, Zona pellucida glycoprotein 3

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Germ cells undergo many events to produce fertile eggs and sperm. During female gametogenesis, oocytes go through primordial follicle assembly, ooplasmic growth, establishment of epigenetic modification, and accumulation of maternal factors; however, a large part of the molecular mechanisms underlying oogenesis remains unknown [1–4]. Although gene knockout is the most common method to investigate gene function, disruption of oogenesis-associated genes occasionally results in embryonic lethality. To overcome this problem, conditional knockout technologies such as the Cre/loxP system have been widely used. However, using this method, mice with oocytes in which the targeted gene is homozygously deleted will not be born until the third generation. As shown in Fig. 1, mice harboring the flox allele and Cre transgenic mice containing the regulatory sequence of an oocyte-specifically expressed gene driving the expression of a Cre recombinase gene are crossed. Following this, mice harboring both flox and Cre alleles are interbred with flox mice. Thus, complete deletion of the targeted gene in the oocytes is achieved in the third generation. The birth rate of these conditional knockout female mice is only 6.25%.

More convenient strategies have also been developed to analyze gene functions in mouse oocytes. Transgenic mice expressing long hairpin RNA using an oocyte-specific gene promoter successfully reproduced the phenotype of *Mos* knockout mice, in which oocytes are parthenogenetically activated [5]. Microinjections of small interfering RNA (siRNA) against *Npm2* and/or *Npm2* mRNA into oocytes in the secondary follicles, and subsequent follicle culture, enabled us to analyze gene function in the oocytes and led to degradation and overexpression of NPM2 protein in the cultured fully grown oocytes (FGOs), respectively [6].

Endogenous microRNAs (miRNAs) are a type of non-coding RNAs that silence genes with complementary sequences; they are found primarily in the intergenic and intronic regions of many genes [7–9]. Intronic miRNA is transcribed as a precursor mRNA of the host gene, and mature miRNA is synthesized by splicing of the host gene and subsequent processing of the primary miRNA. Cytoplasmic mature miRNA forms the RNA-induced silencing complex (RISC) with Argonaute 2, and this RISC complex binds to the mRNAs that are complementary to the miRNA sequences, to inhibit subsequent translation and degrade target mRNAs [10, 11]. Several knockdown approaches using artificially designed miRNA (amiRNA) have been developed *in vivo* and *in vitro* by utilizing this endogenous molecular machinery [12–14]. Miura and colleagues reported that targeted insertion of amiRNA sequences into the intronic site of eukaryotic translation elongation factor 2 (*Eef2*), a constitutively expressed gene, enabled the ubiquitous inhibition of amiRNA-targeting gene expression [15]. This raised the possibility that conditional knockdown could be achieved by amiRNA insertion into a tissue-specifically expressed gene. The development of various methods for gene manipulation during oogenesis contributes to the understanding of maternal factors in oocytes. In the present study, to establish a novel technology for a gene knockdown system during oogenesis, we generated mice in which amiRNAs against the target gene were knocked into the intronic site of the zona pellucida glycoprotein 3 (*Zp3*) gene. We then assessed the knockdown efficiency of the target gene in oocytes.

To establish an oocyte-specific knockdown system using amiRNA,

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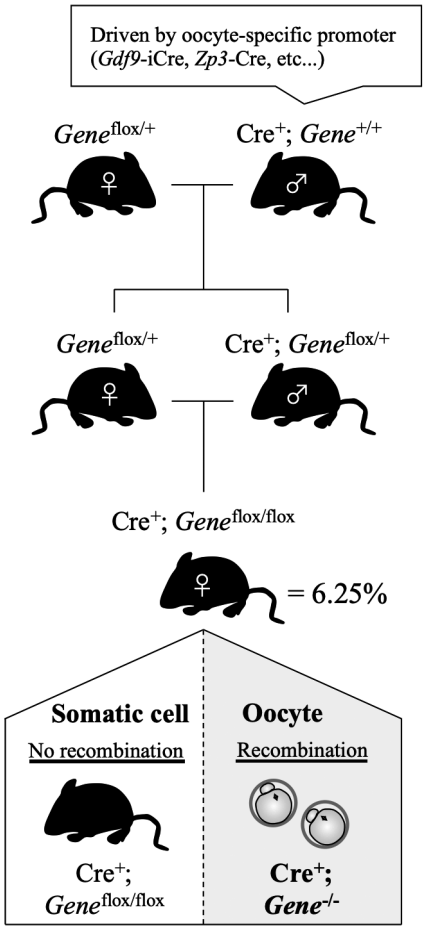

Fig. 1. Schematic representation of breeding strategies in the Cre/loxP-mediated conditional gene knockout. To produce oocyte-specific conditional knockout mice, the floxed heterozygotes ($Gene^{flox/+}$) were crossed with mice possessing oocyte-specifically expressing Cre transgene (Cre^{+}; $Gene^{+/+}$). $Gene^{flox/+}$ and Cre^{+}; $Gene^{flox/+}$ mice were intercrossed to produce conditional knockout female mice in which the oocytes completely lack the functional *Gene* (Cre^{+}; $Gene^{flox/flox}$). This strategy requires repeated breeding for three generations.

we first focused on the *Zp3* gene for the amiRNA knock-in host. As shown in Fig. 2a, *Zp3* expression was drastically upregulated in oocytes at the early growth phase and maintained until the fully grown stage. This indicates that *Zp3* was the most likely candidate for a host gene to achieve knockdown of the target gene throughout oocyte growth.

The amiRNA needs to be processed into a mature form after splicing for knockdown of a target gene. However, whether the *Zp3* intron is an appropriate locus for efficient amiRNA processing and is applicable for clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-mediated genome editing remains unknown. We compared the genomic sequences of *Zp3* among mammals and identified three targeted sites in intron 5 and intron 6, which were less conserved between animal species to avoid interfering transcriptional regulations following the previous study (sites 1–3, Fig. 2b and Table 1) [15]. Knock-in mice were then generated by delivering Cas9-short guided RNA ribonucleoprotein (RNP) complexes and donor single-stranded DNA (ssDNA), composed of both homology arms of each *Zp3* knock-in site and bicistronic amiRNA sequences against enhanced green fluorescence protein (amiR-EGFP #1 and #2), to mouse zygotes (Fig. 2c and Table 2). Genomic sequencing revealed that complete amiR-EGFP sequences were inserted in two of seven founders; others possessed partially lacking amiR-EGFP #2 sequences. Mice in which three amiR-EGFP lines were knocked in at sites 1, 2, and 3, respectively, were used for further experiments, although only 3 bases at the 5′ end of miRNA backbone sequences (Δ3nt) were lacking at site 3. The resultant knock-in mice were crossed with transgenic mice that constitutively express EGFP under control of the synthetic chicken actin gene (CAG) promoter. Subsequently, oocytes were collected from 5-week-old mice possessing both EGFP transgenic and amiR-EGFP alleles (CAG-$EGFP^{+}$; amiR-$EGFP^{+}$) to examine whether EGFP was specifically decreased in FGOs. The results showed that EGFP fluorescence was markedly attenuated in FGOs in all three lines (Fig. 3a and b). This indicates that 1) amiRNA sequences integrated into the *Zp3* introns 5 and 6 were successfully processed into the mature miRNA form to silence the target gene, and 2) the Δ3nt of the miRNA backbone did not have a critical effect on miRNA processing. Most importantly, oocyte-surrounding cumulus cells from CAG-$EGFP^{+}$; amiR-$EGFP^{+}$ mice retained EGFP fluorescence, suggesting that *Zp3* intron-derived amiRNA exhibited oocyte-specific gene knockdown effects (Fig. 3c). Other somatic tissues also showed no EGFP silencing (data not shown).

To investigate the characteristics of *Zp3* intron-derived amiRNA during oocyte growth, we assessed the knockdown effects in oocytes of various sizes: non-growing oocytes (NGOs) at 3 days postpartum (dpp), growing oocytes (GOs) at 14 dpp, and FGOs at 5 weeks of age. EGFP fluorescence was observed in CAG-$EGFP^{+}$; amiR-$EGFP^{+}$ NGOs, similar to that in CAG-$EGFP^{+}$; amiR-$EGFP^{-}$ but started to disappear in CAG-$EGFP^{+}$; amiR-$EGFP^{+}$ GOs larger than 30 μm in a size-dependent manner (Fig. 4a and b). There were no significant differences in the fluorescence intensities of FGO larger than 70 μm between CAG-$EGFP^{+}$; amiR-$EGFP^{+}$ and wild-type mice. The expression levels of both amiR-EGFP #1 and #2 were upregulated in GO and FGO compared with NGO, which was consistent with the *Zp3* expression (Supplementary Fig. 1: online only). Therefore, the method described here is applicable to oocyte-specific gene knockdown after oocyte growth.

Oocytes from *Zp3* heterozygous mutant mice had a thin zona pellucida and showed impaired fertility [16, 17]. However, there were no obvious abnormalities in *Zp3* expression, zona pellucida thickness, ovulation, or fertilization of amiR-$EGFP^{+}$ knock-in oocytes, suggesting that the targeted insertion of amiRNA into the *Zp3* intron did not interfere with transcriptional regulation, including the splicing and translation of host genes, and did not had an unexpected negative influence on oocyte function (Fig. 5a–5d).

Previous studies have shown that the competition of RISC components between siRNA driven by exogenous promoters and naturally produced endogenous miRNA results in toxicity [18, 19]. The amiRNA knockdown strategy also presented the same issue, as transgenic homozygotes expressing amiRNAs driven by exogenous enhancer/promoter were not born [20]. Conversely, we chose the

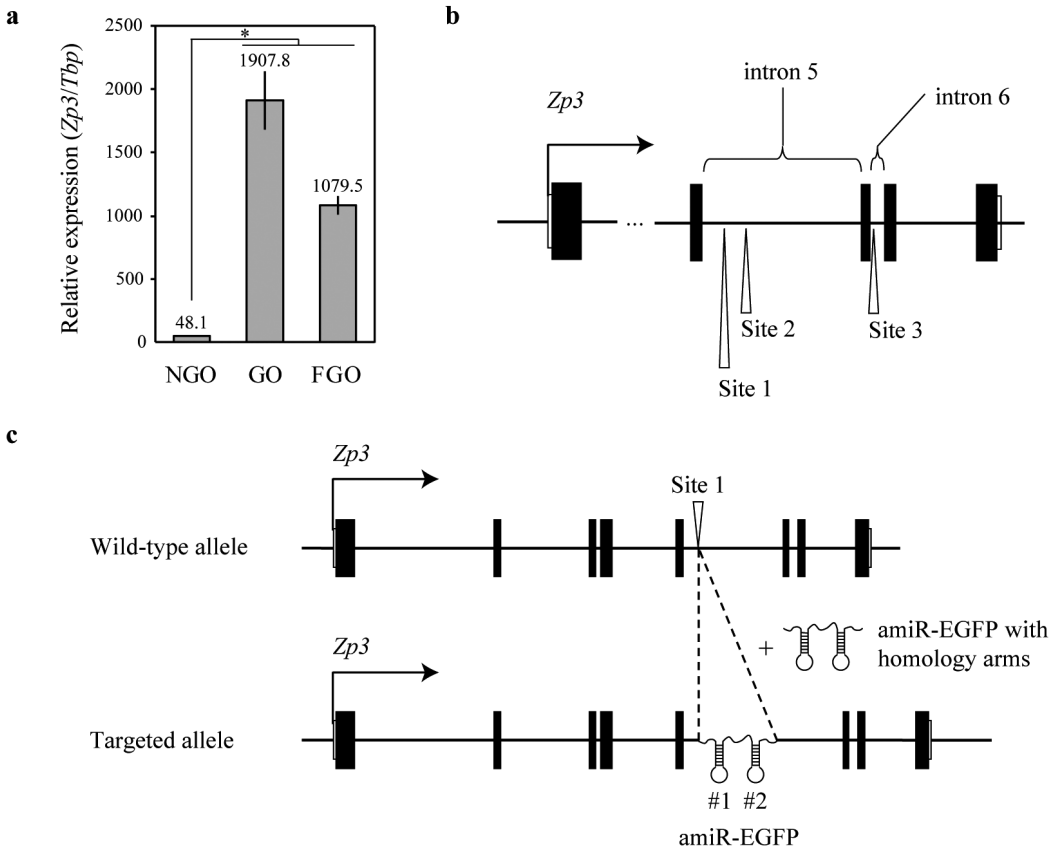

Fig. 2. Establishment of oocyte-specific gene knockdown systems via amiRNA insertion into the *Zp3* intron. a: *Zp3* mRNA expression during oocyte growth. Asterisk indicates a significant difference between NGO and GO-FGO ($n = 3$, Bonferroni's multiple comparison test, $P < 0.05$). b: Targeted sites in *Zp3* introns 5 and 6. c: Schematic representation of CRISPR/Cas9-mediated targeted insertion of amiRNA (amiR-EGFP #1 and #2) into the *Zp3* intron. NGO, non-growing oocyte; GO, growing oocyte; FGO, fully grown oocyte.

Table 1. Information of targeted sequences by CRISPR/Cas9

Targeted site	short guide RNA sequences	Location (chromosome 5)
Site 1 (intron 5)	TTCAGAGGACCAAATCGACA	135,985,953−135,985,972
Site 2 (intron 5)	CCAGGCATGCATGTAGCACG	135,986,038−135,986,057
Site 3 (intron 6)	GTGCCTGTGTGTAGGCACCC	135,987,360−135,987,379

Table 2. Insertion efficiency of amiRNA sequence into the targeted sites of *Zp3* intron

Targeted site	Location	No. of embryos transferred	No. of pups (%)	No. of normally developed (%)	No. of targeted insertion (%)	
					Complete	Partial
Site 1	Intron 5	126	33 (26.2%)	26 (78.8%)	1 (3.8%)	4 (15.4%)
Site 2	Intron 5	64	20 (31.3%)	19 (95.0%)	1 (5.3%)	0 (0%)
Site 3	Intron 6	59	29 (49.2%)	18 (62.1%)	0 (0%)	1 (5.6%)

Parts of pups accidentally died before genomic sequencing (seven for site 1, one for site 2, and 11 for site 3).

endogenous *Zp3* promoter as an amiRNA driver to avoid this issue. Both of the amiR-EGFP$^+$ female (n = 4) and male (n = 7) mice were fertile and exhibited normal phenotype, suggesting that side effects would be lower than in other transgenic RNAi strategies. Furthermore, even if amiRNA-expressing oocytes are infertile due to the knockdown effect of the targeted gene, the amiRNA-knocked-in male mice can survive without any toxicity because of the absence of *Zp3* expression. Thus, our method allows us to stably generate

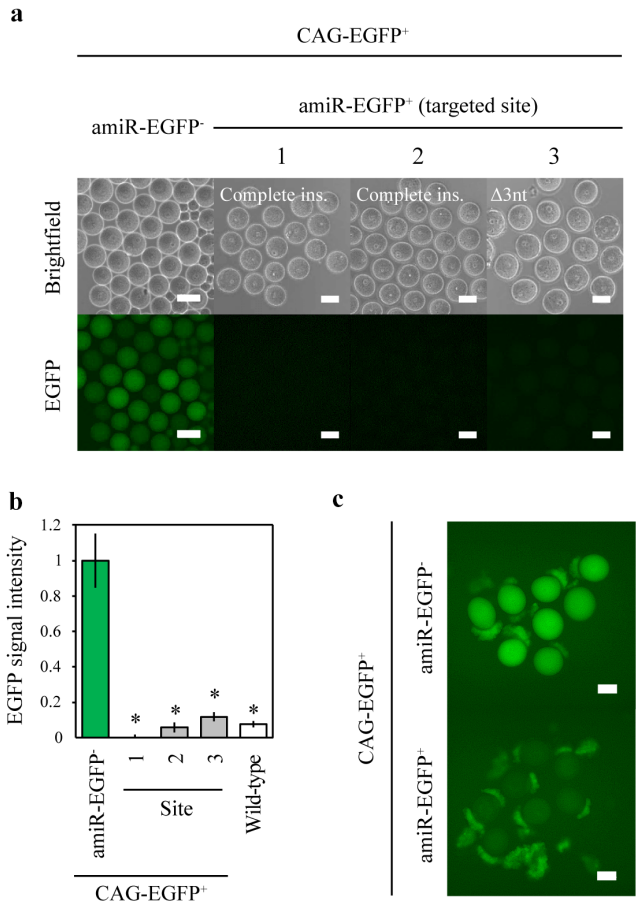

Fig. 3. Conditional knockdown by *Zp3* intron-derived amiRNA in FGO. a: EGFP fluorescence of FGOs from CAG-EGFP$^+$; amiR-EGFP$^+$ mice in each line of targeted site. Transgenic mice of sites 1 and 2 possessed complete inserted alleles, while that of site 3 lacked 3 nucleotides in the amiRNA backbone (Δ3nt). b: Relative signal intensity of EGFP in CAG-EGFP$^+$; amiR-EGFP$^+$ oocytes. Asterisks indicate significant differences between the CAG-EGFP$^+$ positive control oocytes (Bonferroni's multiple comparison test, $P < 0.05$). c: EGFP fluorescence of cumulus cells and FGOs. Scale bar represents 50 μm. FGO, fully grown oocyte.

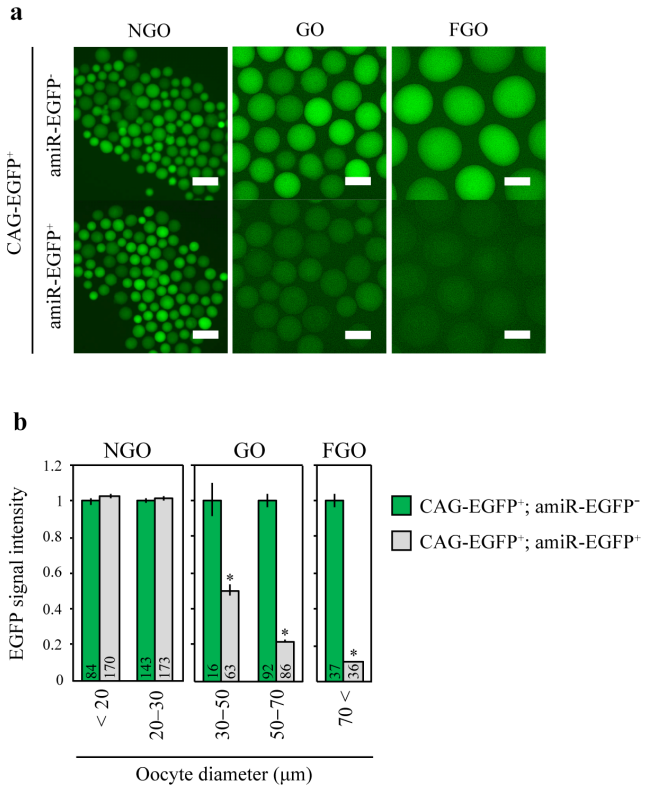

Fig. 4. Size-dependent knockdown efficiencies by amiR-EGFP during oocyte growth phase. a: EGFP fluorescence of oocytes from CAG-EGFP$^+$; amiR-EGFP$^+$ mice throughout oocyte growth. NGO, GO, and FGO were collected from transgenic mice at 3 dpp, 14 dpp, and 5 weeks of age. Scale bar represents 50 μm. b: Oocyte diameter-dependent knockdown effects during oocyte growth. Asterisks indicate significant differences between the CAG-EGFP$^+$ positive control oocytes (T-test, $P < 0.05$). NGO, non-growing oocyte; GO, growing oocyte; FGO, fully grown oocyte; dpp, day postpartum.

conditional knockdown females, at a rate of 25%, with a single mating of a wild-type female and an amiRNA-knocked-in heterozygous male. This is a great advantage from the viewpoint of both strain maintenance and animal welfare.

The present study showed that *Zp3* intron-derived amiRNA is an effective conditional knockdown method in oocytes entering the growth phase. Other than *Zp3*, there are some genes that are expressed at different stages of oocyte growth. Selecting the host gene into which amiRNA is inserted would allow oocyte-specific knockdown at the preferable stage. Furthermore, we have already developed an *in vitro* multiple knockdown system using intron-derived polycistronic amiRNAs by imitating endogenous miRNA clusters in the introns [21]. Targeted insertion of the amiRNA cluster would enable the screening of multiple gene functions in a single transcription unit. Thus, the intronic amiRNA-based gene knockdown method would be one of the options in various scenarios for studying oogenesis.

Methods

Ethics

All animal experiments were conducted under the approval of the Tokyo University of Agriculture Institutional Animal Care and Use Committee (approval number: 2020052), according to the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan.

Mice

We purchased C57BL/6N (B6) and ICR strain mice from CLEA Japan (Tokyo, Japan) and maintained them in the Animal Life Science Center at Tokyo University of Agriculture. B6 mice were sacrificed for *in vitro* fertilization (IVF) experiments; B6 female mice were injected with 5 IU of human chorionic gonadotropin (Gonatropin; ASKA Pharmaceutical, Tokyo, Japan), 48 h after injection with equine chorionic gonadotropin (eCG; Serotropin, ASKA Pharmaceutical) to

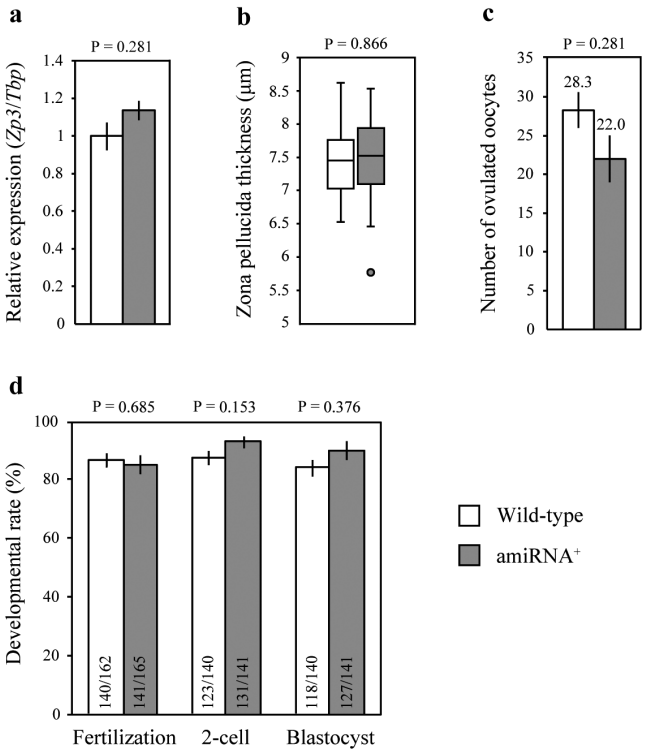

Fig. 5. Characteristics of amiRNA-knocked-in oocytes. a: Expression level of *Zp3* mRNA in amiRNA$^+$ FGO (wild-type, n = 3; amiRNA$^+$, n = 3, T-test). b: Zona pellucida thickness of amiRNA$^+$ FGO (wild-type, n = 37; amiRNA$^+$, n = 36, T-test). c: Number of ovulated metaphase II oocytes from amiRNA$^+$ female mice (wild-type, n = 7; amiRNA$^+$, n = 8, T-test). d: Development of IVF embryos derived from amiRNA$^+$ oocytes (T-test). The number of embryos is shown on each bar. White and gray squares indicate wild-type and amiRNA$^+$ oocytes/embryos, respectively. FGO, fully grown oocyte; IVF, *in vitro* fertilization.

induce superovulation as previously described [22]. To generate amiR-EGFP-knocked-in mice, zygotes were produced by IVF using sperm from B6 male mice in TYH medium (LSI Medience Corp., Tokyo, Japan). amiR-EGFP-knocked-in founder mice were backcrossed with wild-type B6 mice before assessment of the knockdown effect. Following this, to obtain CAG-EGFP$^+$; amiR-EGFP$^+$ female mice, amiR-EGFP-knocked-in female mice were crossed with CAG-EGFP$^+$ male mice, in which EGFP was constitutively expressed [23].

Preparation of donor single-stranded DNA

amiR-EGFP sequences were cloned from pATM (Plasmid #62690, Addgene, Watertown, MA, USA). Passenger strands of amiRNA sequences were designed to lack 2 nucleotides corresponding to positions 12 and 13 of guide strands, as described previously [12]. Targeted plasmid DNAs were synthesized and cloned into the Nb.*Bbv*CI and *Eco*RI sites of the pUCFa vector by Fasmac Co., Ltd. (Atsugi, Japan). Fifty-five nucleotides of the homology arms of the *Zp3* intron for targeted sites 1–3 were bound with the amiR-EGFP sequence. ssDNA was digested with Nb.*Bbv*CI and *Eco*RI; donor ssDNA was subsequently excised and purified using a Long ssDNA Gel Extraction Kit for 3 kb (BioDynamics Laboratory, Tokyo, Japan), following the manufacturer's instructions.

Generation of amiRNA-knocked-in mouse

After 3 h of IVF, zygotes were cultured in KSOMaa medium (Merck, Darmstadt, Germany) for 2 h. Premixed RNP solution (100 ng/μl Guide-it Recombinant Cas9 [TaKaRa Bio, Shiga, Japan], 100 ng/μl Alt-R CRISPR-Cas9 crRNA of sites 1–3 of the *Zp3* intron for each, 100 ng/μl Alt-R CRISPR-Cas9 tracrRNA) and 40 ng/μl donor single-stranded DNA were delivered by electroporation using CUY21EDIT II (BEX, Tokyo, Japan) with a platinum electrode (LF501PT1-10, BEX). Both Alt-R CRISPR-Cas9 crRNA and tracrRNA were purchased from Integrated DNA Technologies (Coralville, IA, USA). Electroporation was performed according to previous reports [24, 25]. After another 24 h incubation in KSOMaa, two-cell embryos were transferred into the oviducts of pseudopregnant ICR female mice.

Genotyping and DNA sequencing

Genomic DNA from founder pups was extracted using genome lysis buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.1% (w/v) SDS, 0.2 mg/ml Proteinase K (Thermo Fisher Scientific, Waltham, MA, USA)]. *Zp3* intronic regions were amplified using KOD One PCR Master Mix (TOYOBO, Osaka, Japan), and visualized with ethidium bromide staining after agarose gel electrophoresis. PCR amplicons were also cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced to determine genomic sequences. DNA sequencing was performed using the BigDye Terminator v3.1 Cycle sequencing Kit (Thermo Fisher Scientific) in an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific). The primer sequences were described in Supplementary Table 1 (online only).

Oocyte collection

NGOs and GOs were collected from 1–3 dpp and 10–14 dpp female mice, respectively. Ovaries were treated with 0.1% collagenase in L-15 medium for 40 min and 0.05% trypsin-0.53 mM EDTA in PBS for 15 min at 37°C. Oocytes and ovarian somatic cells were suspended in M2 medium containing 5 μg/ml cytochalasin B (Sigma-Aldrich Japan, Tokyo, Japan), and the oocytes were isolated using a glass capillary. GOs were treated with 5% Pronase (Sigma-Aldrich Japan) in L-15 medium for 15 min at 37°C to remove the zona pellucida and somatic cells. FGOs at the germinal vesicle stage were obtained from the ovaries of 5-week-old mice 42–46 h after progesterone (Progehormon; Mochida Pharmaceutical) and anti-inhibin serum (AIS; Central Research, Tokyo, Japan) injection [22]. After two daily progesterone injections, AIS was subcutaneously injected 24 h and 48 h after the second progesterone injection. Cumulus cell-oocyte complexes were isolated, and cumulus cells were completely removed by pipetting.

Measurement of EGFP signal intensity in oocytes and of thickness of zona pellucida

Oocytes were collected in 20 μl of M2 medium containing 240 μM dibutyryl cAMP and observed using BZ-X fluorescent microscopy (KEYENCE, Osaka, Japan). Oocyte diameter, thickness of zona pellucida, and EGFP signal intensity were measured using accessory software (KEYENCE) and ImageJ software, respectively [26].

Gene expression analysis

To determine *Zp3* mRNA copy number, total RNA was extracted from oocytes using an RNeasy Micro Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Bio) with Oligo(dT)$_{12–18}$ primer (Thermo Fisher Scientific). Quantitative RT-PCR was performed using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) with the TaqMan Gene Expression Master Mix for *Zp3* (Mm00442176_m1, Thermo Fisher Scientific) and *Tbp* (Mm01277042_m1, Thermo Fisher Scientific). To determine the copy number of targeted genes, partial coding sequences of *Zp3*, which were cloned into the pGEM-T Easy Vector, were used as standards.

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