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Deletion of *Orc4* during oogenesis severely reduces polar body extrusion and blocks zygotic DNA replication[†]

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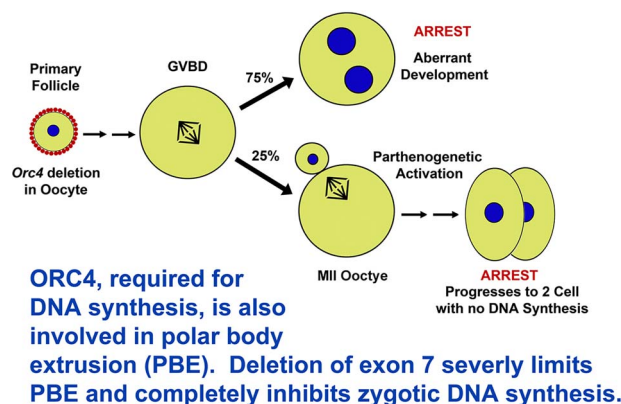
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Abstract

Origin recognition complex subunit 4 (ORC4) is a DNA-binding protein required for DNA replication. During oocyte maturation, after the last oocyte DNA replication step and before zygotic DNA replication, the oocyte undergoes two meiotic cell divisions in which half the DNA is ejected in much smaller polar bodies. We previously demonstrated that ORC4 forms a cytoplasmic cage around the DNA that is ejected in both polar body extrusion (PBE) events. Here, we used ZP3 activated Cre to delete exon 7 of *Orc4* during oogenesis to test how it affected both predicted functions of ORC4: its recently discovered role in PBE and its well-known role in DNA synthesis. *Orc4* deletion severely reduced PBE. Almost half of *Orc4*-depleted germinal vesicle (GV) oocytes cultured in vitro were arrested before anaphase I (48%), and only 25% produced normal first polar bodies. This supports the role of ORC4 in PBE and suggests that transcription of the full-length *Orc4* during oogenesis is required for efficient PBE. *Orc4* deletion also abolished zygotic DNA synthesis. Fewer *Orc4*-depleted oocytes developed to the metaphase II (MII) stage, and after activation these oocytes were arrested at the two-cell stage without undergoing DNA synthesis. This confirms that transcription of full-length *Orc4* after the primary follicle stage is required for zygotic DNA replication. The data also suggest that MII oocytes do not have a replication licensing checkpoint as cytokinesis progressed without DNA synthesis. Together, the data confirm that oocyte ORC4 is important for both PBE and zygotic DNA synthesis.

Summary Sentence ORC4 functions in polar body extrusion during oocyte maturation and fertilization, then in DNA replication in the zygote. Conditional knock-out of *Orc4* in primary follicles causes a reduction in PBE and complete inhibition of DNA synthesis in the zygote.

Graphical Abstract



Keywords: ORC4, meiosis, polar body exclusion, DNA replication, DNA replication licensing

Introduction

Oocyte growth in mice progresses to the formation of primordial follicles by the first day after birth and to fully formed preovulatory follicles by day 22 (Supplementary Figure 1)

[1]. DNA synthesis is completed before the primary follicle stage, and the oocytes are 4C (4C refers to the chromosome complement of the cell, which indicates that there are four copies of each chromosome) with homologous chromosomes

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already paired [2]. Upon ovulation, the oocyte progresses through the first meiotic division when it asymmetrically divides into the first polar body that contains half of the chromosomes but only a fraction of the cytoplasm (this process is called polar body extrusion or PBE). The daughter oocyte enters immediately into the second metaphase, MII, with a 2C complement of chromosomes retained on a meiotic spindle. Upon fertilization, the oocyte completes its last meiotic division, which is also asymmetric, yielding the second polar body and the zygote. Each polar body degrades shortly after it is formed. Transcription is maintained by the oocyte until just before maturation and is shut down in mature MII oocytes [3]. Some transcription occurs in the zygote after DNA replication, but most transcription resumes at the two- or four-cell stage.

We have previously demonstrated that PBE in both meiotic divisions is accompanied by the formation of an ORC4 cage that surrounds the chromatin at anaphase that is expelled in the polar body but does not surround the chromatin that remains in the oocyte [4–6]. The ORC4 protein is part of the origin recognition complex (ORC) that identifies the origins of replication in virtually all eukaryotic species examined from yeast to mammals [7, 8]. The ORC contains six proteins that are highly conserved throughout evolution. Only licensed origins are replicated, and loss of the ORC causes inhibition of DNA replication in most eukaryotic cells. The two different roles of ORC4 in PBE and DNA synthesis are complementary and mutually exclusive. During PBE, ORC4 forms a cytoplasmic cage that surrounds the chromosomes that will be extruded in both meiotic divisions [4, 5]. This sequestration of ORC4 in the cytoplasm may be part of the mechanism that allows the cell to proceed through a second cytokinesis without an intervening DNA replication to complete meiosis. When ORC4 resumes its role in DNA synthesis, it migrates into the nucleus as expected [5].

Our finding that ORC4 formed a cage around the chromosomes that are expelled [4–6] was surprising given ORC4's normal function in DNA replication. However, there is some evidence that ORC proteins play other roles in cell division [9, 10], so our identification of ORC4's potential new role in meiosis was at least not without precedent. Mammalian ORC4 has an independent DNA-binding domain [11], and ORC4 directs the localization of the entire ORC complex to the origins in yeast [12].

Our previous work provided evidence that ORC4 was required for PBE. For example, we demonstrated that when ORC4 peptides were injected into MII oocytes prior to the formation of the second ORC4 cage, the polymerization of ORC4, and therefore the cage formation, was prevented and PBE was inhibited [4]. We also demonstrated that when decondensed sperm chromatin, which is normally not surrounded by an ORC4 cage, is induced to be extruded as a pseudo polar body, an ORC4 cage does form around the paternal DNA that is extruded. These data demonstrated a functional relationship between the formation of the ORC4 cage and PBE. Given that the role of ORC4 in PBE was so unexpected, we sought to develop a direct, genetic strategy to provide new evidence that ORC4 is required for PBE. This was challenging because ORC4 is required for DNA replication, and therefore it is expected to be embryonically lethal if deleted.

The aim of this research was to determine how deletion of *Orc4* during oogenesis would affect both functions of ORC4, in PBE and in DNA replication, during oocyte development. The two functions occur temporally apart (PBE is

completed after the last DNA replication before meiosis and before the first zygotic DNA replication). We therefore took advantage of a well-developed model to disrupt genes using a *Zp3* promoter-driven *Cre* (*Zp3-Cre*) [13–15] to inactivate the *Orc4* gene specifically during oogenesis at the primary follicle stage (Supplementary Figure 1).

The *Zp3-Cre* model has been used by several laboratories to inactivate genes that affect oocyte development, with up to 100% efficiency for removing the floxed sites [15], which demonstrates that ongoing transcription during oogenesis after the primary follicle stage is required for proper oogenesis. For example, McGuinness et al. [16] inactivated the serine/threonine protein kinase BUB 1(F) resulting in mis-segregation at meiosis I. Ploutarchou et al. [17] inactivated glycoprotein-N-acetylgalactosamine 3-b-galactosyltransferase 1 (*C1galt1*), a gene that controls core 1-derived O-glycans in oocytes and found that the cumulus cell–oocyte complex was smaller. Using the *Zp3-Cre* model, Shi et al. [18] deleted α -1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase (*Mgat1l*) that is crucial for the synthesis of proteoglycans in developing oocytes and embryonically lethal in homozygous mice. They found that mutant oocytes had thinner zona pellucidas than controls and did not contain ZP1, ZP2, or ZP3. Together, these past studies support that transcription after the primary follicle stage is essential for oogenesis and that the *Zp3-Cre* model can effectively achieve inactivation of the genes involved.

Here, we excised the floxed exon 7 of the *Orc4* gene with *Zp3-Cre* during oogenesis to better understand the roles of ORC4 in PBE and zygotic DNA synthesis. We demonstrated that PBE is severely restricted upon *Orc4* deletion and confirmed ORC4's role in the first round of DNA synthesis in the embryo.

Methods

All experimental protocols using mice in this work were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Hawaii and followed the ethical procedures for the care and use of experimental animals.

Breeding scheme to obtain mice with *Orc4* deleted in oogenesis

We obtained frozen sperm from the C57BL/6NTac-*Orc4* <tm1a(EUCOMM)Wtsi>/IcsOrl mouse strain from the European Mouse Mutant Archive (EMMA). This strain has a gene trap on exon 7 of the *Orc4* gene. These sperms were injected into C57BL/6J mice, then bred to flippase mice to release the gene trap, then bred to homogeneity until a colony of *Orc4*^{Flox/Flox} mice were obtained. Deletion of both copies of the *Orc4* gene is embryonically lethal (unpublished data from gene trap results of EMMA at <https://www.mousephenotype.org/data/genes/MGI:1347043>), presumably because ORC4 is required for DNA synthesis (Supplementary Figure 2). Therefore, we used a well-established breeding scheme that allows for the production of homozygous *Orc4*^{Flox/Flox} mice that have at least one copy of the *Zp3-Cre* gene to allow *Cre* to inactivate the *Orc4* gene during oogenesis [13, 19]. To verify that the promoter to *Zp3* activated *Cre* only in oocytes and as early as the primary follicle stage of oogenesis as has been reported [20], we first crossed a female mouse containing a tdTomato red fluorescent protein (RFP) reporter

gene with a male *ZP3-Cre^{+/+}* mouse. Oocytes in primordial follicles from the female progeny did not express RFP but most oocytes in primary and secondary follicles did express RFP (Supplementary Figure 3A–D). The surrounding somatic cells had no fluorescence. Germinal vesicle (GV) oocytes from adult females also showed tomato RFP expression only in the oocytes but not in the surrounding cumulus cells (Supplementary Figure 3E and F). These data indicated that *ZP3-Cre* expression was limited to the oocytes and not the surrounding cumulus cells as expected.

We next bred female *Orc4^{Flox/Flox}* mice with male *ZP3-Cre^{+/+}* to create *Orc4^{Flox/wt}; ZP3-Cre^{+/-}* mice [13, 19]. In the final step, male *Orc4^{Flox/wt}; ZP3-Cre^{+/-}* mice were bred with female *Orc4^{Flox/Flox}* mice to create four different genotypes, only one of which, *Orc4^{Flox/Flox}; ZP3-Cre^{+/-}* (referred to as *Orc4*-CKO), is a conditional knockout of *Orc4* because it activates Cre during oogenesis to inactivate the full-length *Orc4* gene by excising exon 7. Female *Orc4*-CKO mice were infertile as expected (explained next), so this breeding scheme had to be repeated for every experiment.

Preparation of GV oocytes, gametes, and embryo culture

Spermatozoa were obtained from B6 or transgenic male mice (8–10 weeks old). A caudal epididymis was dissected, sperm mass released into CZB [21] and incubated for 1 h at 37°C under 5% CO₂ in air to allow the spermatozoa to disperse in the medium. The spermatozoa suspension for intracytoplasmic sperm injection (ICSI) was mixed with polyvinylpyrrolidone (PVP)-saline (0.9% NaCl containing 10% w/v PVP [360 kDa; ICN Biochemicals]). To obtain mature MII oocytes, mature females, 8–10 weeks old, were induced to superovulate by injections of 5 IU eCG and 5 IU hCG, given 48 h apart. Oviducts were removed 14–15 h after the injections of hCG and placed in light mineral oil for embryo culture (Irvine, catalog number 9305). The cumulus cells were released from the oviducts into 0.1% bovine testicular hyaluronidase/CZB medium for 10 min to disperse cumulus cells. The cumulus-free oocytes were washed and kept in CZB at 37°C under 5% CO₂ in air. These oocytes were used for immunocytochemistry (ICC) and ICSI. To collect immature metaphase I (MI) and anaphase I (AI) oocytes, females were injected with 5 IU of eCG. Ovaries were removed 24 h thereafter and placed in HEPES-CZB in a Petri dish. The biggest follicles were broken to release GV-stage oocytes. GV oocytes with surrounding cumulus cells were placed in CZB drops under mineral oil and cultured for 2 h at 37°C, 5.0% CO₂. After 2 h, cumulus cells were removed by pipetting. The oocytes that underwent germinal vesicle breakdown were collected and cultured for further 3, 9, and 12 h to obtain MI, AI, and MII stages, respectively. Some of these oocytes were then used for ICC.

Parthenogenetic activation of MII oocytes

MI oocytes were incubated with CZB-free, Ca²⁺-supplemented 10 mM SrCl₂ for 2 h. The oocytes were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and cultured for up to 2 days to follow their progression to the stages up to the two-cell stage.

Intracytoplasmic sperm injection

ICSI was carried out as described by Ward and Yanagimachi [22]. ICSI was performed using Eppendorf micromanipulators (Micromanipulator TransferMan, Eppendorf, Germany)

with a piezoelectric actuator (PMM Controller, model PMAS-CT150; Prime Tech, Tsukuba, Japan). A single sperm head was sucked, tail first, into the injection pipette and moved back and forth until the head-midpiece junction (the neck) was at the opening of the injection pipette. The head was separated from the midpiece by applying one or more piezo pulses. An oocyte was held to the holding pipette at the 9 o'clock position. After discarding the midpiece and tail, the sperm head was redrawn into the pipette and injected immediately into an oocyte. After ICSI, oocytes were cultured in CZB at 37°C under 5% CO₂ in air.

Isolation of primordial, primary, and secondary follicles

To collect these oocytes, 7-day-old pups from mating *ZP3-Cre^{+/+}* males with tdTomato females were used to collect ovaries. Isolated ovaries were placed in Accumax solution (catalog number A7089; Sigma Aldrich) in a Petri dish for 10 min. The follicles were manually isolated in HEPES-CZB using 30 G needles and categorized into primordial, primary, and secondary follicles according to their size and morphology [23]. These follicles were used to analyze the expression of RFP under fluorescent microscopy.

Antibodies

Polyclonal goat anti-ORC4 was obtained from Santa Cruz Biotechnology (C-15, catalog number sc-19,726; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies included Alexa Fluor 488 rabbit antigoat (Invitrogen, Grand Island, NY). Click-it EdU Alexa Fluor 488 kit (catalog number C10350, Invitrogen) was used to detect DNA replication.

Immunocytochemistry

Oocytes and embryos were cultured in CZB until they reached the desired stage after ICSI or culture. The oocytes or embryos were fixed in 2% paraformaldehyde for 30 min at room temperature. After fixing, embryos were washed three times with 0.1% Tween/PBS (PBSw) for 10 min. Cells were permeabilized in 0.5% Triton X-100 for 15 min, then the cells were washed three times with PBSw containing 10% volume of 5% bovine serum albumin (BSA). Cells were blocked with 5% BSA for 1 h at room temperature, then incubated in primary antibody at 1:50 dilution overnight at 4°C. On the next day, the cells were again washed three times with PBSw containing 10% volume of 5% BSA, then incubated in secondary antibody at 1:1000 dilution at room temperature for 1 h. Cells were then washed three times with PBSw containing 10% volume of 5% BSA and finally the cells were mounted with ProLong Gold antifade reagent with DAPI (catalog number P-36931; Invitrogen). The staining oocytes or embryos were analyzed with an FV1000-IX81 confocal microscope from Olympus using the Fluoview v. 2.1 software. For each stage of oocyte or embryonic development, at least 20 embryos were examined by ICC, and the results were only reported if they were consistent in all embryos.

Aphidicolin treatment

MI oocytes collected from *Orc4*-CKO, and wild-type females were activated with 10 mM of SrCl₂ for 3 h. The activated oocytes were then incubated with Aphidicolin (3 μL of 1 mg Aphidicolin/mL) overnight at 37°C in a humidified atmosphere of 5% CO₂ in air to inhibit DNA synthesis. These

oocytes were then used for DNA synthesis assays (catalog number C10350; Invitrogen) and then mounted with ProLong Gold antifade reagent with DAPI (catalog number P-36931; Invitrogen). DNA synthesis was analyzed with an FV1000-IX81 confocal microscope from Olympus using the Fluoview v. 2.1 software.

Quantitative reverse transcriptase-polymerase chain reaction

Total RNA from 100 *Orc4*-CKO MII oocytes was extracted and purified using the TRIzol and Trace kit (Thermo Fisher Scientific, catalog number A33250). cDNA was then synthesized from this total RNA by reverse transcription of polyadenylated RNA using superscript reverse transcriptase IV following the manufacturer's protocols (Thermo Fisher Scientific, catalog number 18090050). Then, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using SYBR Green PCR Master Mix on an ABI Step-OnePlus machine (Applied Biosystems, Carlsbad, CA). RT-PCR reactions were performed at 95°C for 10 min followed by 35 PCR cycles (10 s at 95°C and 60 s at 60°C). Three types of PCR reactions were performed: (1) Primers designed at exons 7 and 9 were used to amplify *ORC4* variant 1 (v1-*Orc4*) from wild-type and *Orc4^{Flox/Flox}* oocytes; (2) primers designed at exons 6 and 8 were used to amplify v1-*Orc4* mRNA from wild-type, *Orc4^{Flox/Flox}*, and *Orc4*-CKO oocytes; and (3) primers designed at exons 6 and 7* (7* is a variant exon) were used to amplify *ORC4* variant 2 (v2-*Orc4*) (Supplementary Table 1). All of the reactions were performed in triplicate per assay, and β -actin was included in every PCR reaction as a loading control. The different values in PCR cycles for β -actin for a given experimental sample were subtracted from the mean Δ Ct of the reference samples (*Orc4^{Flox/Flox}*) ($\Delta\Delta$ Ct) [24, 25]. The quantification of the *ORC4* knockdown values were further normalized to $\Delta\Delta$ Ct values of β -actin. Primers are listed in Supplementary Table 1.

Single oocyte PCR

A single *Orc4*-CKO MII oocyte or an *Orc4^{Flox/Flox}* oocyte was added to a PCR tube containing 4 μ L of GNTK buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris, pH 8.5, 0.45% Triton X100, 0.45% Tween 20) supplemented with 100 μ g/mL proteinase K. These tubes were incubated at 55°C for 2.5 h with a Bio-RAD C100 Touch thermo cycler to extract total DNA. These DNA tubes were directly used to perform PCR using a KOD hot start DNA polymerase kit (Millipore/Sigma, catalog number 71086). The primers used were CKO-F and CKO-R (Supplementary Table 1). The PCR amplification reactions were performed using a PCR Bio-RAD C100 Touch thermocycler. The PCR cycle protocol consisted of 2 min hold at 95°C, followed by 35 cycles of 10 s at 98°C, 30 s at 60°C, and 2 min at 72°C, then with a final step for 5 min at 72°C. The PCR products were used to run with 1% agarose supplement with ethyl bromide. The gel was imaged using a Fujifilm LAS-3000 imager.

Image presentation

All micrograph images were constructed by placing the raw images on a single figure. Immunocytochemical images were all then enhanced together so that relative comparisons can be seen. Phase-contrast images were enhanced separate together but separately from the fluorescent images.

Statistical analysis

For statistical analysis comparing the development of *Orc4*-CKO and *Orc4^{Flox/Flox}* GV oocytes to MII oocytes, percentages were transformed to arcsine values (Supplementary Table 3), then compared by the Student *t*-test [26, 27].

Results

Deletion of *Orc4* in *Orc4*-CKO mice occurs by the MII stage

Inactivating a floxed gene using Zp3-Cre has been reported to be capable of being 100% effective [15]. We tested the efficiency of the Zp3-Cre in our hands by single-oocyte PCR. We used a set of primers for two loci just outside the loxP sites flanking exon 7 that are present in all genotypes (CKO-F and CKO-R, Supplementary Figure 2). We performed single oocyte PCR on 27 MII oocytes from 4 *Orc4^{Flox/Flox}* mice, which have exon 7, and 92 oocytes from 6 *Orc4*-CKO mice, in which we expected exon 7 to be removed by Zp3-Cre. We did not obtain PCR products from all oocytes, but every PCR product was the expected size (Supplementary Figure 4, Supplementary Table 2). We examined 92 *Orc4*-CKO oocytes and 51 had PCR products that were consistent with exon 7 being excised, as expected. The other 41 had no PCR products, indicating that our technique was not sensitive enough to amplify DNA from every oocyte. However, none of the positive PCR products contained exon 7. Conversely, 19 of the 27 *Orc4^{Flox/Flox}* oocytes that had PCR products were all positive for exon 7 as expected. None of *Orc4*-CKO oocytes retained exon 7, and all of the *Orc4^{Flox/Flox}* oocytes were positive for exon 7. Fewer *Orc4*-CKO oocytes had PCR products than *Orc4^{Flox/Flox}* oocytes (55.4% vs. 70.4%, respectively), but this was not surprising given that the *Orc4*-CKO MII oocytes were more poorly developed and may have had degenerated DNA. We conclude that Zp3-Cre was remarkably effective at removing *Orc4* exon 7 during oogenesis.

Depletion of *Orc4* transcripts in *Orc4*-CKO mice

The PCR data described above demonstrated that the exon 7 of the *Orc4* genes were undetectable in MII *Orc4*-CKO oocytes. This deletion causes a frame-shift in the remainder of the *Orc4* gene resulting in an inactive *Orc4* transcript. However, it is possible that some complete *Orc4* mRNA could have been transcribed before the gene was inactivated during oogenesis and stored for use in the MII stage or later. We therefore performed RT-PCR on MII oocytes from *Orc4^{Flox/Flox}* and *Orc4*-CKO mice to determine the levels of detectable transcripts. The major *Orc4* mRNA variant (v1-*Orc4*, NCBI reference sequence: NM_011956.3) contains all 14 exons and is translated to the full-length *ORC4* protein (Supplementary Figure 5). There is another splice variant of *Orc4* known as variant 2 (v2-*Orc4*) that contains the first 6 exons of *Orc4* and a different exon 7 (NCBI reference sequence: NM_001177213.1) [28]. We expected that *Orc4*-CKO oocytes would not produce v1-*Orc4* transcripts but might produce a transcript that did not contain exon 7 and exons 8–14 in a frameshift (Supplementary Figure 5). We also expected that *Orc4*-CKO oocytes could still produce v2-*Orc4* (Supplementary Figure 5). We therefore tested for the presence of mRNAs using forward and reverse primers located in exons

Table 1. Female *Orc4*-CKO mice are infertile, but males are fertile. Female *Orc4*-CKO mice were mated with normal B6 males (wild-type), and no pups were obtained. Male *Orc4^{Flox/Flox};Cre^{+/−}* mice were able to sire pups when mated with normal females.

Male	Female	Number of matings	Number of pups/litter Mean + SD
wt	wt	4	8.5 + 2.6
wt	<i>Orc4</i> -CKO	4	0
<i>Orc4^{Flox/Flox};Cre^{+/−}</i>	wt	2	8 + 1.4

6 and 8, respectively, which would amplify both wild-type v1-*Orc4* and *Orc4*-CKO mRNAs, and using forward and reverse primers located in exons 7 and 9, respectively, which would only amplify wild-type v1-*Orc4* transcript. In *Orc4*-CKO oocytes, we observed the expression of transcripts amplified with both primer pairs, but transcripts amplified with the primers located in exons 7 and 9 were at very low levels (0.15-fold) whereas transcripts amplified with the primers located in exons 6 and 8 were approximately half lower (0.58-fold) when compared to the control (Supplementary Figure 6). Interestingly, the v2-*Orc4* transcript increased by $5.21 + 3.25$ -fold in *Orc4*-CKO mice as compared to the controls. These data suggested that the production of normal, full-length *Orc* mRNA was severely reduced in *Orc4*-CKO mice but increased in v2-*Orc4*.

Fertility of *Orc4*-CKO mice

When *Orc4*-CKO mice were mated with wild-type mice, female *Orc4*-CKO mice were completely infertile. No pups were born from four mating attempts (Table 1). Male *Orc4^{Flox/Flox};Cre^{+/−}* mice were fertile when mated with wild-type females.

Orc4-CKO GV oocytes have reduced polar body extrusion

We isolated 221 GV oocytes from 10 adult *Orc4*-CKO mice and compared their progression in vitro with 84 oocytes from 3 *Orc4^{Flox/Flox}* control mice. These mice had the *Orc4^{Flox/Flox}* genotype (Supplementary Figure 2B) in all cells, except for the oogenic cells, which had the *Orc4^{−/−}* phenotype (Supplementary Figure 2C). GV oocytes have 4C DNA, before the first polar body extrusion releases half the DNA. Normal GV oocytes progress to the MII oocyte stage within 12 h of culture, which includes the extrusion of the first polar body. However, *Orc4*-CKO GV oocytes did not progress normally in culture. We found that only 25% of *Orc4*-CKO GV oocytes progressed to the MII stage (Figures 1A–D and 2) whereas almost half (48%) were arrested at the GV, MI, or AI stages (Figures 1A–C and 2, Supplementary Figures 7 and 8). The remaining *Orc4*-CKO GV oocytes progressed to aberrant stages including oocytes with two or more pronuclei, or oocytes with one pronucleus and one polar body (Figure 1E and F). *Orc4*-CKO oocytes had some ORC4 staining (Figure 1C and H), suggesting that some ORC4 that was already present before *Zp3-Cre* is activated might have remained in the oocyte during development. To test this, we stained primary follicles, the stage at which *Zp3* is first activated, for cytoplasmic ORC4 and found that it was present

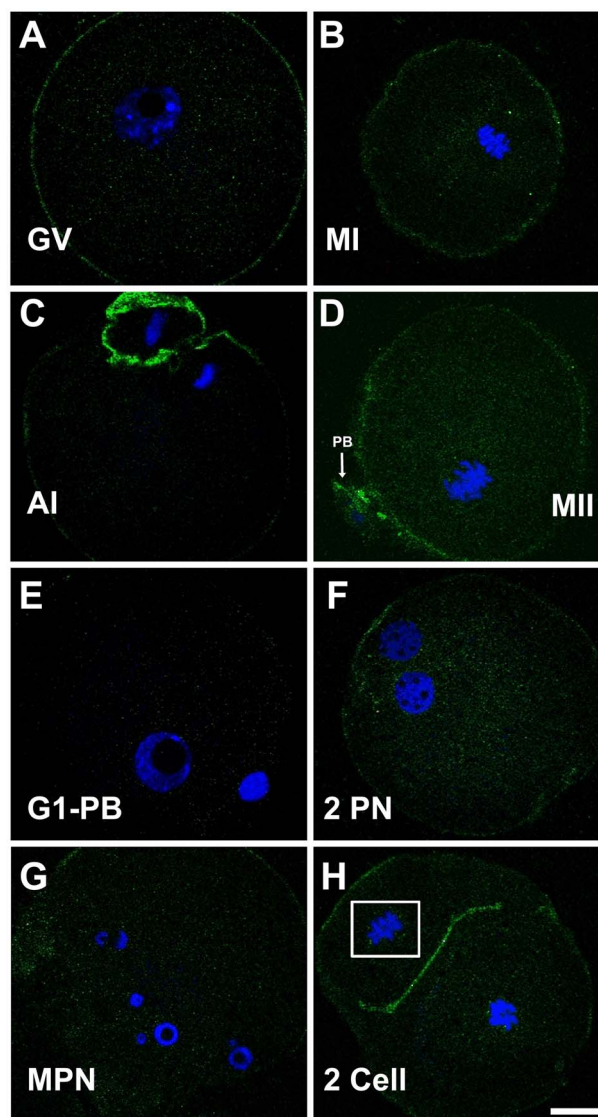


Figure 1. Development of *Orc4*-CKO GV oocytes in vitro. GV oocytes from *Orc4*-CKO females were isolated and allowed to mature in vitro for 24 h. This resulted in some normal and abnormal developmental stages which were stained for ORC4 using ICC (green) and counterstained with DAPI for DNA visualization (blue). (A–C) Oocytes that were arrested at normal stages of maturation in vitro, but did not progress. (A) An oocyte that was arrested at the GV stage. Some ORC4 staining is present just below the oolemma. (B) An oocyte that was arrested at MI. (C) An oocyte that was arrested at anaphase I, with a strong ORC4 staining pattern. (D) Some oocytes progressed to the MII stage, which was the normal point of progression. (E–H) Abnormal oocytes that represent aberrant stages of the first meiotic division. (E) An oocyte in which the second metaphase plate failed to develop and resolved into a pronucleus (larger nucleus) and a polar body (G1-PB). (F) An oocyte where the metaphase I plate resolved into two pronuclei with no polar body (2-PN). (G) An oocyte in which the metaphase plate resolved into multiple pronuclei with no polar body (MPN). (H) An oocyte in which the first meiotic division resulted in two cells (2-Cell) (Note: One nucleus was in a different focal plane from the rest of the image and is shown in a white box, inset). All images are shown at the same magnification (bar = 20 μ m). For phase-contrast and single-stained images, see Supplementary Figure 7.

(Figure 3). Nevertheless, only little ORC4 was retained and our immunofluorescence data demonstrated that deletion of exon 7 of the *Orc4* gene caused severe disruption in normal PBE.

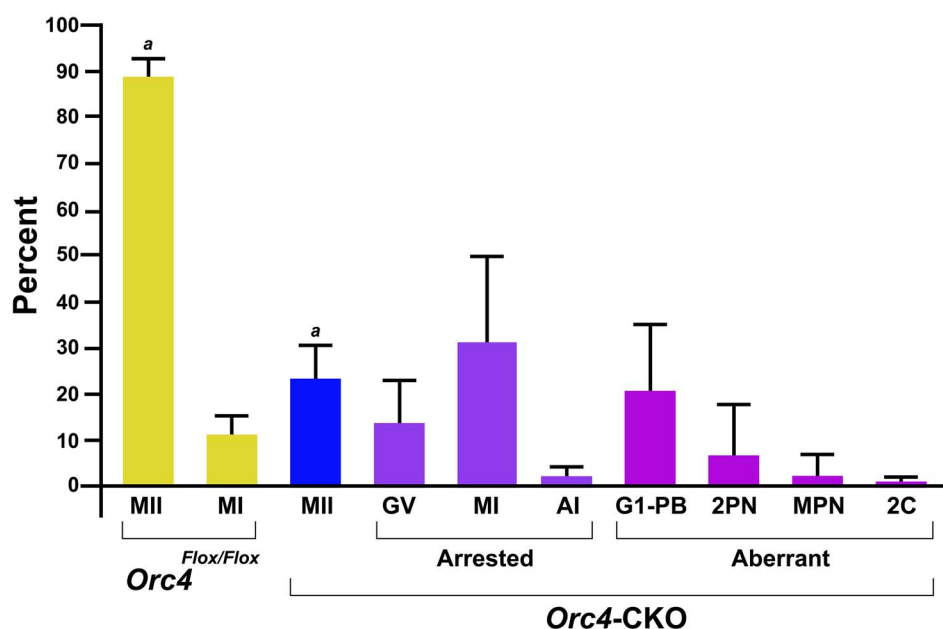


Figure 2. Maturation of *Orc4*-CKO GV oocytes in vitro. A total of 221 GV oocytes were isolated from 10 *Orc4*-CKO females and 84 GV oocytes from *Orc4^{Flox/Flox}* controls and cultured for 24 h. The developmental stage that each GV oocyte reached after 24 h was recorded by immunocytochemical analysis. None of the aberrant stages was noted in the controls. All values are means of oocytes from different mice, and error bars are standard deviations. The difference in the development of *Orc4*-CKO and *Orc4^{Flox/Flox}* GV oocytes against MII oocytes was highly significant ($P = 0.0001$, noted with an a above the bars). The numbers of oocytes in each category for each mouse are shown in [Supplementary Table 4](#). (MI, metaphase I; MII, metaphase II; GV, germinal vesicle; AI, anaphase I; G1-PB, an oocyte with one pronucleus and one polar body; 2PN, an oocytes with two pronuclei; MPN, an oocytes with more than two pronuclei; 2C, two-cell embryo).

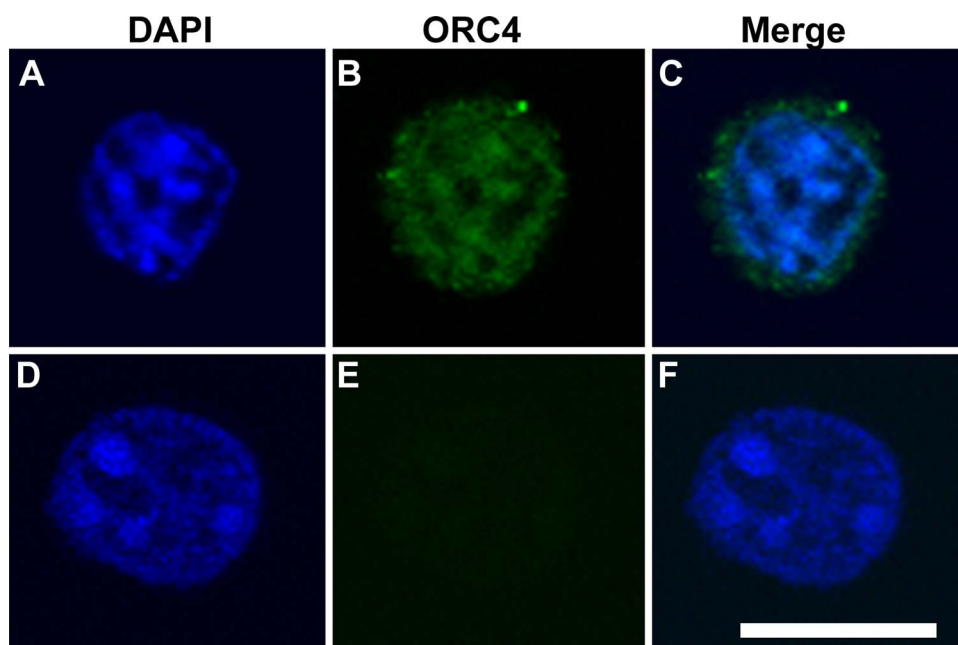


Figure 3. Primary follicles contain cytoplasmic ORC4. Primary follicles were isolated from 7-day-old pups and stained for the presence of ORC4. (A–C) DAPI, ORC4 stained, and merged fluorescent images, respectively, of a primary follicle stained with ORC4 antibody. (D–F) As in (A–C) except that no primary antibody was included. Bar = 10 μ m.

Orc4-CKO MII oocytes progress to the two-cell stage without DNA synthesis

When *Orc4*-CKO females were stimulated with eCG and hCG to superovulate, they produced fewer MII oocytes than controls ([Table 2](#)). Most of the *Orc4*-CKO MII oocytes that were stimulated with SrCl₂ to activate parthenogenesis

progressed to the two-cell stage, but none of these two-cell embryos developed any further ([Table 3](#)). These embryos had extremely condensed nuclei that appeared to have degraded DNA ([Figure 4A and B](#); for an example of a normal parthenogenetic embryo's two-cell nuclei, see [Figure 5A](#)). Examining 58 oocytes from three different *Orc4*-CKO mice, we found

Table 2. Average number of MII oocytes from control and *Orc4*-CKO mice. The number of MII oocytes that were obtained from control (*Orc4^{Flox/Flox}*) and *Orc4*-CKO mice were counted. The differences between the total number of oocytes per mouse between the *Orc4^{Flox/Flox}* and *Orc4*-CKO was statistically significant ($P = 0.0001$, Student *t*-test).

Mouse	Number of mice	Number of oocytes	Number of oocytes/mouse Mean + SD
<i>Orc4^{Flox/Flox}</i>	12	388	32.3 + 5.6*
<i>Orc4</i> -CKO	26	436	17.4 + 4.6*

* $P = 0.0001$.

that an average of 72.4% had either unequal distribution of DNA or no DNA in one or both cells (Supplementary Table 5). In some cases of these aberrant embryos, there was no DNA in one of the two cells (Figure 4C and D) or no DNA detectable in either embryonic cell, even when DNA was visible in the residual polar body (Figure 4E and F). This suggests that parthenogenetically activated *Orc4*-CKO two-cell embryos had degraded DNA.

The progression to the two-cell stage in *Orc4*-CKO oocytes was inconsistent with the second of the two ORC4 functions we studied, its role in DNA synthesis. ORC4 is thought to be required for DNA synthesis but progression from G1 through mitosis usually requires DNA replication. We therefore tested whether the *Orc4*-CKO MII oocytes that progressed to the two-cell stage did so without replicating their DNA, or if, as has been shown in some cancer cells [29], they could replicate DNA in the absence of the ORC proteins. Control parthenogenetic zygotes incubated for 24 h in EdU had robust evidence of DNA replication in both nuclei at the two-cell stage (Figure 5A–C). However, *Orc4*-CKO two-cell embryos did not have evidence for any EdU incorporation during this time period even in those embryos that retained visible levels of DNA (Figure 5G–I). The progression of *Orc4*-CKO embryos to the two-cell stage in the absence of DNA synthesis was in contrast to previous work in which zygotes that were cultured in the presence of the DNA polymerase inhibitor aphidicolin were arrested at the one-cell stage [30]. We repeated this previously published experiment as a control for this study, illustrating that cytokinesis of normal mouse zygotes is inhibited by the inhibition of DNA synthesis with aphidicolin (Figure 5D–F). We next tested the remote possibility that aphidicolin might also arrest cytokinesis in parthenogenetically activated *Orc4*-CKO MII oocytes as a secondary effect unrelated to its direct inhibition of DNA polymerase. We activated *Orc4*-CKO MII oocytes in the presence of aphidicolin and assessed them after 24 h. We found that parthenogenetically activated *Orc4*-CKO MII oocytes still progressed to the two-cell stage in the presence of aphidicolin (Figure 4J–L). These data suggest that inhibition of DNA synthesis by ORC4 depletion occurs by a different mechanism than that of aphidicolin treatment. Note that in aphidicolin-treated *Orc4*-CKO two-cell embryos, the nuclei are not condensed and the DNA complement appears to be normal (compare Figure 5G with Figure 5J). These results have implications for cell cycle control in the zygote as discussed next.

ICSI does not rescue *Orc4*-depleted oocytes

There was an unlikely possibility that the paternal genome could rescue the first round of DNA synthesis by either

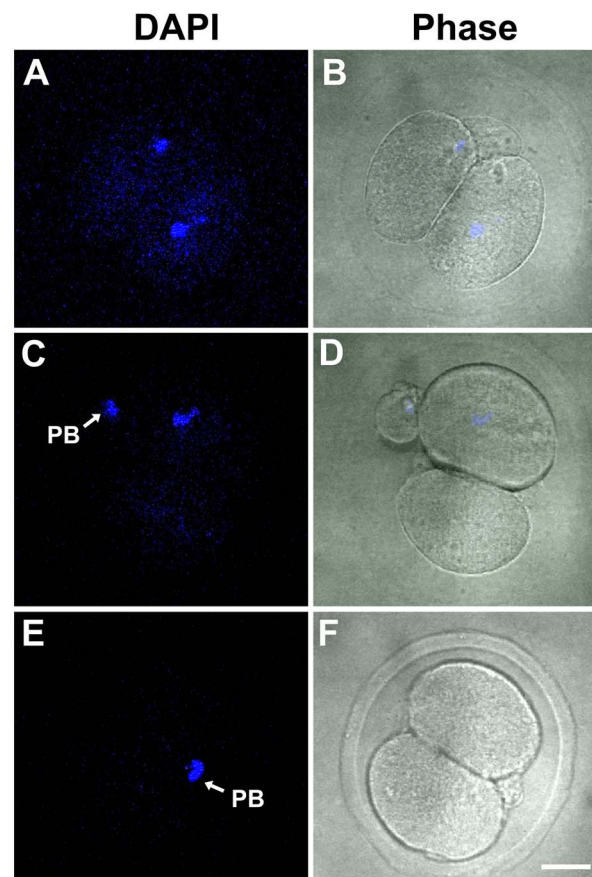


Figure 4. Distribution of DNA in two-cell embryos from *Orc4*-CKO parthenogenesis. *Orc4*-CKO MII oocytes were collected and activated with SrCl₂ and imaged after 24 h. This figure shows three examples with DAPI staining to reveal the DNA (A, C, and E) and the corresponding phase-contrast images (B, D, and F, respectively). (A, B) An embryo that had roughly equal distribution of DNA in both blastomeres even though the DNA appears degraded or dispersed. (C, D) An embryo which had visible DNA in one cell but none in the other. Note that the DNA in the polar body is visible. (E, F) An embryo in which there was no DNA visible in either blastomere. Normal parthenogenotes should have clearly visible nuclei in both blastomeres (Figure 5A). Bar = 20 μ m.

supplying ORC4 itself, or by supplying a functional copy of the *Orc4* gene that could be transcribed in the zygote [31, 32]. We injected *Orc4*-CKO MII oocytes with wild-type sperm and incubated the zygotes with EdU for 8 h to detect DNA synthesis. *Orc4^{Flox/Flox}* showed robust DNA synthesis (Figure 6A–C) whereas *Orc4*-CKO zygotes had no evidence of replication (Figure 6D–F). These data suggest that the zygote depends on ORC4 supplied by the oocyte for the first round of DNA synthesis and that a normal *Orc4* gene supplied by the sperm cell cannot rescue zygotic DNA synthesis in the absence of ORC4. The data also demonstrated that the infertility of the female *Orc4*-CKO mice was not due to the inability to activate when fertilized or to decondense the sperm nucleus because these zygotes formed normal pronuclei (Figure 6D).

Discussion

We undertook this study to examine how the two functions of ORC4, its role in PBE, and its requirement for DNA replication, were affected by inactivation of the *Orc4* gene

Table 3. Progression of *Orc4*-CKO MII Oocytes. MII oocytes were obtained from *Orc4*^{Flox/Flox} and *Orc4*-CKO mice and *Orc4*-CKO mice, activated with SrCl₂ to progress parthenogenetically and cultured for 2 days. The progression was monitored by phase-contrast microscopy

Mouse	Number of mice	Number of MII oocytes	One-cell		Two-cell		Four-cell	
			Ave + SD	%	Ave + SD	%	Ave + SD	%
<i>Orc4</i> ^{Flox/Flox}	2	62	26.5 + 2.1	85.5	24.5 + 0.7	92.5	24.5 + 0.7	100
<i>Orc4</i> -CKO	11	216	17.7 + 3.9	90.3	12.5 + 3.3	70.8	0.0	0

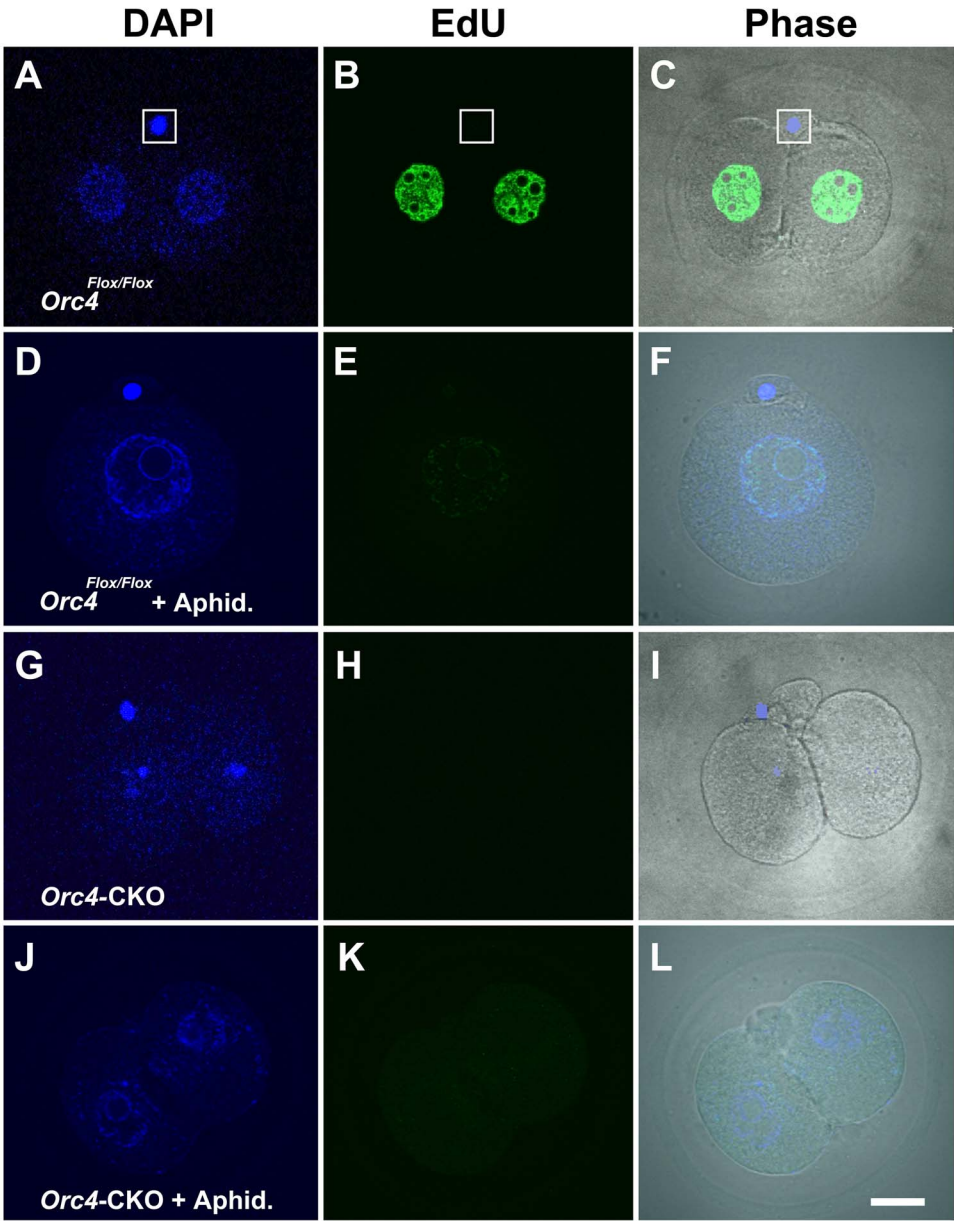


Figure 5. Activated *Orc4*-CKO oocytes progress to the two-cell stage without DNA synthesis. *Orc4*^{Flox/Flox} and *Orc4*-CKO MII oocytes were activated to progress through embryonic development by treatment with SrCl₂, supplemented with or without aphidicolin (DNA polymerase inhibitor) treatment in the presence of EdU and immunostained 24 h later. Panels on the left show DAPI staining, panels in the middle show EdU incorporation for DNA synthesis, and panels on the right show merged images with phase contrast. (A–C) A normal, activated oocyte incorporates DNA (B) and divides into two cells (C). (D–F) When normal MII oocytes are incubated with aphidicolin shortly after activation, DNA synthesis is inhibited (E) and the cells are arrested at G1 and do not divide (F). (G–I) Activated *Orc4*-CKO oocytes progress to the two-cell stage with no visible DNA synthesis. (J–L) Treatment of *Orc4*-CKO oocytes does not prevent their progression to two-cell embryos. All images are shown at the same magnification. Bar = 20 μ m.

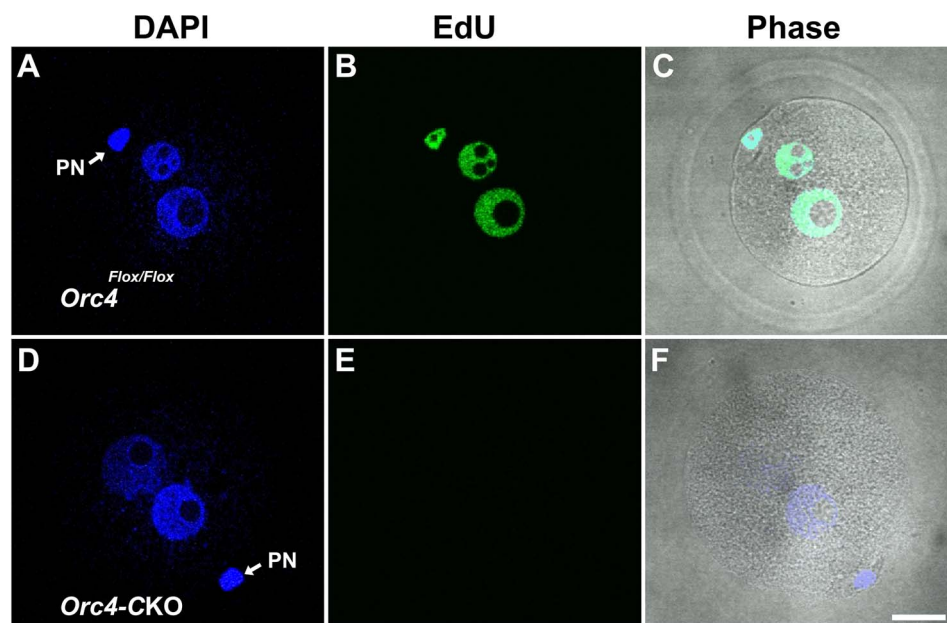


Figure 6. ICSI does not rescue DNA synthesis in *Orc4*-CKO embryos. Control and *Orc4*-CKO MII oocytes were injected with wild-type spermatozoa containing wild-type copies of the *Orc4* gene and incubated with EdU for 8 h. (A–C) A control *Orc4*^{Flox/Flox} embryo showing incorporation of EdU into both pronuclei and the polar body (PB) (panel B). (D–F) An embryo produced from an *Orc4*-CKO oocyte injected with a normal spermatozoon. No evidence of DNA incorporation is seen (E). All images are shown at the same magnification. Bar = 20 μ m.

during oogenesis. To achieve this, we used ZP3 activated Cre to delete exon 7 of *Orc4* during oogenesis. We had to use this approach because *Orc4* deletion is embryonically lethal as our finding that *Orc4*-CKO embryos could not replicate DNA support. We found that ORC4's role in PBE was partially compromised by removing *Orc4* exon 7, and DNA replication in the zygote was completely inhibited. The Zp3-Cre system is effective because transcription in the oocyte continues even after the DNA is in the 2N/4C stage with two sister copies of each chromosome condensed with its homologue in synaptonemal complexes. Several studies have used the Zp3-Cre system of inactivating floxed genes during oogenesis with phenotypes evident during oocyte development indicating that transcription and translation are still active in the oocyte during oogenesis [15, 17, 19].

Fertility of *Orc4*-CKO mice

Orc4-CKO female mice were infertile. This was expected because the original gene-trapped mice, *Orc4*^{tm1a(EUCOMM)Wtsi}, (Supplementary Figure 2A) were embryonically lethal when the deletion was homozygous. In our *Orc4*-CKO mice (Supplementary Figure 2B), exon 7 was removed only in oocytes, after the last oocyte DNA synthesis, by ZP3-Cre (Supplementary Figure 2C). Thus, we expected normal development of both male and female first-generation *Orc4*-CKO because the ZP3-Cre would not be activated in zygotes created by mating female *Orc4*^{Flox/Flox} mice with male ZP3-Cre mice. Male *Orc4*-CKO mice were fertile, as expected, because ZP3-Cre was never activated. Female *Orc4*-CKO mice were expected to be infertile because DNA synthesis was inhibited in the fertilized zygote due to the lack of ORC4. But even if DNA synthesis was restored, it is also probable that the impaired PBE due to the reduction in ORC4 would have also reduced fertility.

ORC4 and polar body extrusion

One major goal of this work was to test whether deletion of *Orc4* during oogenesis would inhibit PBE as we have predicted from previous experiments [4, 5]. We found that when *Orc4* was deleted during oogenesis, normal PBE was reduced by 75% in GV oocytes cultured in vitro (Figure 2). Although 25% of GV oocytes progressed to normal MII oocytes in vitro, there was only a 54% reduction in the number of MII oocytes obtained from *Orc4*-CKO ovaries (Table 2). This difference in the number of MII oocytes actually produced in *Orc4*-CKO females and the progression of GV oocytes in culture may be related to the stresses of in vitro culture that made the GV oocytes more susceptible to the partial loss of ORC4 available for PBE [33, 34]. Thus, these data support our hypothesis that ORC4 is required for normal PBE. There are three nonmutually exclusive possible explanations for the fact that *Orc4* deletion did not fully inhibit PBE. First, although we demonstrated that the *Orc4* gene was successfully targeted by the MII stage (Supplementary Figure 4), it is possible that intact *Orc4* mRNA was still being transcribed through the GV stage. This would have allowed the oocyte to produce full-length cytoplasmic ORC4 after the primary follicle stage, and this protein could have contributed to PBE. Second, it is possible that enough cytoplasmic ORC4 had already been produced by the follicle stage and the protein retained during oogenesis to allow for some PBE. This is supported by continued visible staining of ORC4 in GV oocytes. Third, we have previously shown that the v2-ORC4 variant of ORC4 protein is present in the ORC4 cage along with v1-ORC4 during PBE [4] and it is possible that contribution of v2-ORC4 was enough to allow for the inefficient PBE we observed. Our RT-PCR data show that the level v2-*Orc4* mRNA is increased in *Orc4*-CKO oocytes suggesting that the oocyte might be compensating for the loss of full length v1-ORC4. If any of these possibilities is true, then it is unlikely that a transgenic

mouse model that deletes *Orc4* will allow for the complete inhibition of PBE. We are currently testing whether deletion of an earlier exon that would abrogate both variants of *Orc4* will inhibit PBE more clearly.

ORC4 is required for the first zygotic DNA synthesis

The second function of ORC4 that we examined was its more established role in DNA synthesis. This function was completely inhibited in embryos resulting from oocytes from *Orc4*-CKO females. Our data clearly demonstrate that ORC4 is required for the first round of DNA synthesis in the mouse embryo. Although this was expected from the known function of the ORC in the licensing of DNA replication origins [7, 35, 36], recent data on some mammalian systems have demonstrated that it is possible for cells to replicate DNA in the absence of core components of this protein complex. Shibata et al. [29] demonstrated that certain human cancer cell lines can replicate DNA when *ORC1* and *ORC2* are deleted. This suggests that mammalian cells do have alternate mechanisms for replicating DNA. Okano-Uchida et al. [37] created a mouse that had a floxed *ORC1* gene and crossed this mouse with *Sox2*-Cre that deactivated the gene during early development. These authors showed that *ORC1* was required for embryonic development, but in this case zygotic embryos replicated efficiently, most likely due to the fact that *SOX2* becomes activated after the two-cell stage. These *Orc1*^{-/-} embryos developed to the blastocyst stage but not much further. Trophoblast lineages, however, continued to replicate DNA. When these mice were crossed with *Albumin-Cre*, adult livers retained the ability to regenerate, replicating by endoreduplication. Together, these studies demonstrated that mammalian cells do not always require the intact ORC for DNA replication. The ZP3-Cre model to inactivate *Orc4* allowed demonstrating that the intact ORC is required in the first zygotic DNA synthesis.

ORC4 is part of the complex that recruits CDC6 to the licensing site [8], so the lack of ORC4 would be expected to inhibit licensing, and, thereby, the initiation of DNA synthesis. The progression of activated oocytes to the two-cell stage in the absence of ORC4 was not prevented by the inability of these embryos to license DNA for replication. This has two potential implications. The first is that mouse zygotes do not have a functional DNA licensing checkpoint. The p53-dependent checkpoint arrests cells in either S-phase or G1, depending on the cell type, until the chromatin is fully licensed [38–40]. The fact that *Orc4*-CKO zygotes were not arrested in the absence of DNA licensing suggests that zygotes do not have a licensing checkpoint. Secondly, the data support a “brake” model for the progression through the cell cycle. Recent studies have demonstrated that the transition from S-phase to M-phase is probably due to an inhibition of PLK1 during the S-phase with a gradual release of inhibition during G2 that culminates in mitosis (reviewed in [41]). Lemmens et al. [42] demonstrated that in mammalian cells, when licensing was inhibited by specifically degrading CDC6, cells progressed directly to mitosis without DNA synthesis (these authors used a cell line in which p53 was deleted to prevent activation of the licensing checkpoint so that they could analyze the S/G2/M transitions). CDK-controlled progression through the cell cycle is temporarily halted during S-phase and renewed when S-phase is completed [42]. The *Orc4*-CKO zygotes were not arrested in G1, presumably because of the absence of the licensing checkpoint, then continued to

progress through to mitosis because of the lack of the brake in S-phase that normally temporarily halts cell cycle progression. Normal oocytes that were treated with aphidicolin would have had normal licensing as aphidicolin directly inhibits DNA polymerase [43], which would not have disrupted DNA replication licensing. The brake for cell cycle progression is activated but never released because DNA synthesis is never completed. In the *Orc4*-CKO zygotes, aphidicolin did not arrest the cell cycle because DNA synthesis was never initiated. These results therefore support the interpretation that the lack of intact ORC4 prevented licensing in the oocytes, and this was the cause of the inhibition of DNA synthesis.

Conclusions

In this work we have shown that mutating the *Orc4* gene specifically during oogenesis causes reduced PBE and a complete inhibition of DNA synthesis in the zygote. Although the two functions of ORC4 are temporally separate, both are affected by *Orc4* deletion. However, ORC4's role in DNA synthesis is completely inhibited whereas its role in PBE is only partially inhibited by *Orc4* conditional deletion. This suggests that residual ORC4 is partially able to support PBE, but newly transcribed ORC4 is required for DNA synthesis. Mouse zygotes also appear to not have a functional replication licensing checkpoint and proceed to the two-cell stage without DNA synthesis, thus supporting the brake model for cell cycle progression.

Authors' contribution

HN, HW, AU, YY, BF, and WSW each provided substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data. HN and WSW drafted the article and revised it. BF and YY edited the manuscript. All the authors have provided a final approval for the manuscript to be publication ready.

Supplementary material

Supplementary material is available at *BIOLRE* online

Data availability

All original data in this publication are available upon request to the corresponding author. There are no large databases associated with this work.

Conflict of interest

None of the authors declare any conflicts of interest.

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