Oocyte stage-specific effects of MTOR determine granulosa cell fate and oocyte quality in mice

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MTOR (mechanistic target of rapamycin) is a widely recognized integrator of signals and pathways key for cellular metabolism, proliferation, and differentiation. Here we show that conditional knockout (cKO) of *Mtor* in either primordial or growing oocytes caused infertility but differentially affected oocyte quality, granulosa cell fate, and follicular development. cKO of *Mtor* in nongrowing primordial oocytes caused defective follicular development leading to progressive degeneration of oocytes and loss of granulosa cell identity coincident with the acquisition of immature Sertoli cell-like characteristics. Although *Mtor* was deleted at the primordial oocyte stage, DNA damage accumulated in oocytes during their later growth, and there was a marked alteration of the transcriptome in the few oocytes that achieved the fully grown stage. Although oocyte quality and fertility were also compromised when *Mtor* was deleted after oocytes had begun to grow, these occurred without overtly affecting folliculogenesis or the oocyte transcriptome. Nevertheless, there was a significant change in a cohort of proteins in mature oocytes. In particular, down-regulation of PRC1 (protein regulator of cytokinesis 1) impaired completion of the first meiotic division. Therefore, MTOR-dependent pathways in primordial or growing oocytes differentially affected downstream processes including follicular development, sex-specific identity of early granulosa cells, maintenance of oocyte genome integrity, oocyte gene expression, meiosis, and preimplantation developmental competence.

oocyte-expressed MTOR | granulosa cell | Sertoli-like cells | meiosis | female infertility

Nongrowing primordial oocytes surrounded by flattened somatic cells form primordial follicles that develop perinatally, persist throughout mammalian female reproductive life, and serve as the source of growing follicles. Primordial oocytes are, therefore, the storage form of oocytes and are sometimes considered quiescent or dormant, although processes needed to maintain oocyte viability obviously must be sustained for prolonged periods—for years in some species. Other potential activities of primordial oocytes that may be necessary for fertility are unknown. Oocyte and follicular development initiates with the transition of the primordial oocytes to an active growing stage and the proliferation of the surrounding somatic cells, the granulosa cells. Then, together, the growing oocyte and proliferating granulosa cells embark on a complex and coordinated program of oocyte and follicular development that culminates in ovulation of a mature egg (1–3). The lineage, differentiation, and function of granulosa cells, particularly those closely associated with oocytes, is dependent upon paracrine factors secreted by oocytes (4–8), although it is not clear whether the presence of the oocyte participates in sustaining the sex-specific developmental and functional identity of granulosa cells (9–11).

MTOR (mechanistic target of rapamycin) is widely recognized as an integrator of signals and pathways key for cellular metabolism, proliferation, and differentiation (12). It controls myriad life processes by linking extra- and intracellular cues from nutrients, stress, growth factors, and hormones, and its dysfunction is associated with an increasing number of pathological conditions, including cancer, obesity, type 2 diabetes, and neurodegeneration (12–14). In mouse ovarian follicles, the MTOR pathway is selectively activated in cumulus cells, the granulosa cells surrounding oocytes, before ovulation induction, and this specific activation is partially attributable to oocyte-suppressing expression of *Ddit4l*, a negative regulator of MTOR (15). This oocyte-enabled activation of MTOR is crucial for the development and survival of both cumulus cells and oocytes (15). *Mtor* is also robustly expressed in oocytes; however, its function, especially its specific roles in the control of coordinated development and function of oocytes and granulosa cells, was unknown. Here, we deleted *Mtor* specifically in oocytes at two different developmental stages: primordial and growing oocytes. Both conditional knockouts (cKOs) caused infertility, demonstrating the crucial role of oocyte-expressed *Mtor* in female reproduction; however, the resultant oocyte and granulosa cell phenotypes differed in these two cKOs, reflecting changing functions of the MTOR-dependent pathways during oocyte development.

Significance

MTOR (mechanistic target of rapamycin), an integrator of pathways important for cellular metabolism, proliferation, and differentiation, is expressed at all stages of oocyte development. Primordial oocytes constitute a nonproliferating, nongrowing reserve of potential eggs maintained for the entire reproductive lifespan of mammalian females. Using conditional knockouts, we determined the role of MTOR in both primordial and growing oocytes. MTOR-dependent pathways in primordial oocytes are not needed to sustain the viability of the primordial oocyte pool or their recruitment into the cohort of growing oocytes but are essential later for maintenance of oocyte genomic integrity, sustaining ovarian follicular development, and fertility. In growing oocytes, MTOR-dependent pathways are required for processes that promote completion of meiosis and enable embryonic development.

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Data deposition: RNA-seq data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (datasets GSE98497 and GSE114126); proteomics data have been deposited in the ProteomeXchange Consortium (dataset PXD006408).

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Results and Discussion

Oocyte-Specific Knockout of *Mtor* Compromises Oocyte Quality and Female Fertility in Mice. MTOR was expressed in both the oocytes and granulosa cells of all stages of follicles being examined (*SI Appendix*, Fig. S1 *A* and *B*). To assess the function of oocyte-expressed MTOR, we produced *Mtor* oocyte-cKO mice by crossing female mice carrying the conditional allele of *Mtor* ($Mtor^{tm1.2Koz}$) with male transgenic (Tg) mice [Tg(*Gdf9*-icre)5092Coo and Tg(*Zp3*-cre)93Knw mice] expressing the transgene for Cre recombinase specifically in oocytes at either primordial or early growing stages (Fig. 1*A*) (16). We refer to the *Gdf9*-CRE–mediated cKO in primordial oocytes as "*Mtor*-GcKO" and the *Zp3*-CRE–mediated cKO in growing oocytes as "*Mtor*-ZcKO." Both immunofluorescence (IF) and Western blot analyses showed that MTOR was nearly undetectable in primordial oocytes of *Mtor*-GcKO ovaries and at all subsequent oocyte stages (Fig. 1*B* and *SI Appendix*, Fig. S1*C*). In *Mtor*-ZcKO ovaries, MTOR was present at normal levels in primordial oocytes, but only trace levels, probably residual protein from primordial follicles, were detected in growing oocytes (Fig. 1*B* and *SI Appendix*, Fig. S1*C*). These data confirmed the effectiveness of the specific deletion of MTOR in the primordial and growing oocytes, respectively, by these two cKOs. Furthermore, Western blot analysis revealed that both MTOR and its downstream activities were barely detected in the fully grown oocytes (FGOs) of both cKO mice (Fig. 1*C*), thus indicating the efficient abrogation of the MTOR pathways.

Fertility testing revealed that, unlike WT female mice that produced about six litters per mouse during 8–10 mo of breeding, with an average of about six mice per litter, *Mtor*-GcKO females were completely infertile (Fig. 1*D*). *Mtor*-ZcKO females were nearly infertile (Fig. 1*D*): Only one of the tested *Mtor*-ZcKO females conceived during the entire fertility testing period, and that female produced only one litter with only one pup (Fig. 1*D*). Therefore, oocyte MTOR is indispensable for female fertility in mice. *Mtor*-GcKO and *Mtor*-ZcKO females ovulated an average of 3 and 30 oocytes, respectively, compared with an average of 43 ovulated oocytes in WT females (Fig. 1*D*). Only about 20% of the cKO-ovulated oocytes underwent successful fertilization and development to the two-cell stage after in vitro fertilization (IVF) with normal sperm (Fig. 1*D*), and 10% or less of the fertilized cKO-oocytes developed to blastocysts in culture (Fig. 1*D* and *SI Appendix*, Fig. S2*A*). The latter result was repeated with the oocytes that underwent in vitro maturation (*SI Appendix*, Fig. S2 *B* and *C*). In sum, the ovulation rate was dramatically reduced when *Mtor* was deleted at the primordial oocyte stage but was reduced only slightly when *Mtor* was deleted at the growing oocyte stage. Oocyte developmental competence was severely compromised in both *Mtor*-GcKO and *Mtor*-ZcKO oocytes (*SI Appendix*, Table S1).

Defective Development of the Follicles and Granulosa Cells in *Mtor*-GcKO but Not *Mtor*-ZcKO Ovaries. Follicular development was quantified to explore the basis for the reduced ovulation rate, particularly in

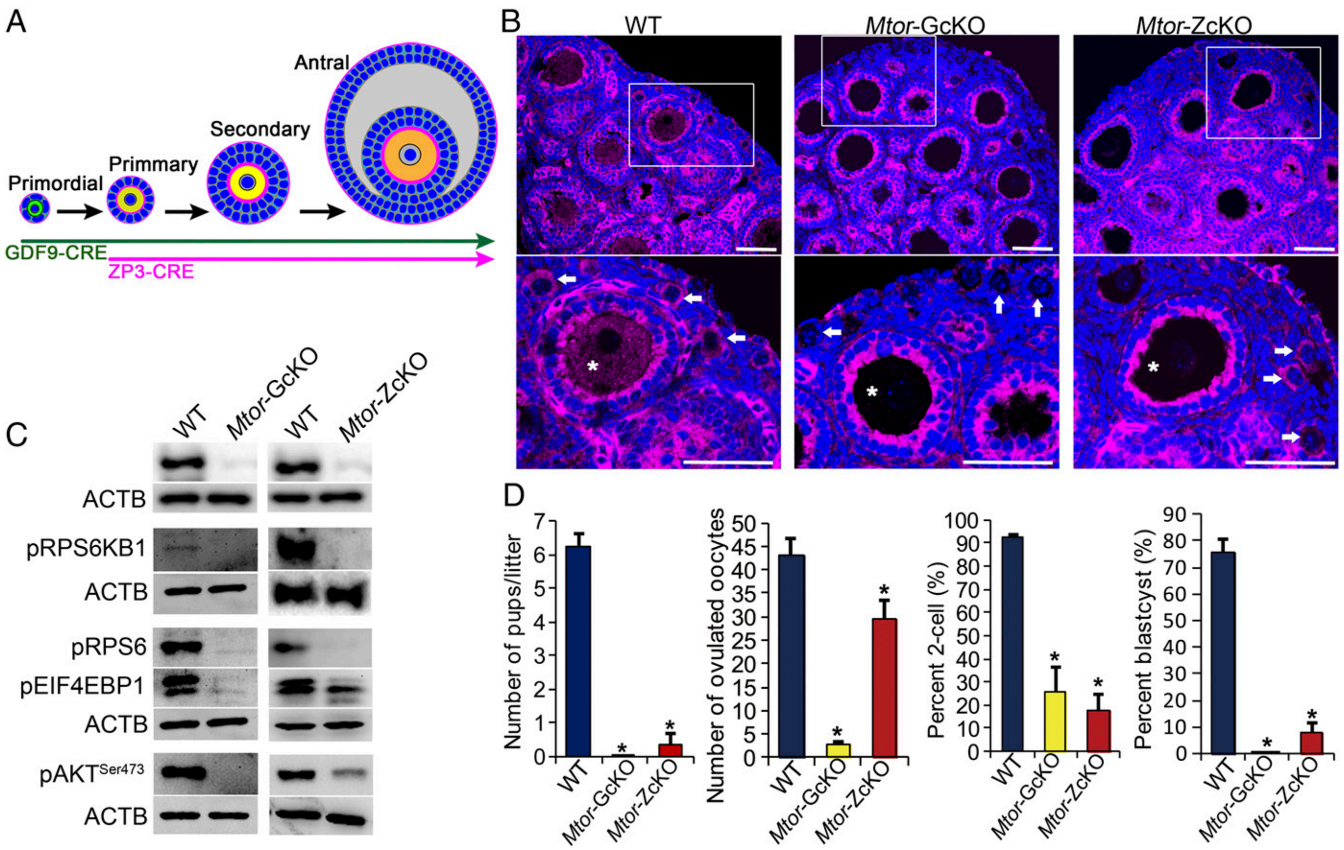

Fig. 1. Infertility and compromised oocyte quality in cKO mice. (*A*) Schematic illustration of the stages at which GDF9-Cre and ZP3-Cre are expressed. Expression of GDF9-Cre and ZP3-Cre starts in primordial and growing oocytes, respectively. (*B*) IF staining of MTOR in 21-d-old WT, *Mtor*-GcKO, and *Mtor*-ZcKO ovaries. MTOR and DNA are stained in magenta and blue, respectively. Arrows point to oocytes in primordial follicles; asterisks indicate growing oocytes. (Scale bars, 50 μm.) (*C*) Western blot analysis of the expression of MTOR and the activated form of its major downstream effectors—pRPS6KB1, pRPS6, pEIF4EBP1, and p-AKT^{Ser473}—and the internal control ACTB in WT, *Mtor*-GcKO, and *Mtor*-ZcKO FGOs. (*D*) Number of pups born and number of oocytes ovulated by WT and *Mtor*-cKO females and the rate of two-cell and blastocyst formation by ovulated WT and *Mtor*-cKO oocytes after IVF. $^*P < 0.05$, compared with the WT or control by student's *t* test. Data represent the mean ± SEM.

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Mtor-GcKO females. Neither of the cKOs affected the number of primordial follicles when quantified in the ovaries of 21-d-old mice (*SI Appendix*, Fig. S3); thus MTOR-dependent pathways were not necessary for the survival of the primordial oocyte pool, at least to this age. However, there were fewer large secondary and more primary follicles in the ovaries of 21-d-old *Mtor*-GcKO prepubertal mice (Fig. 2*A* and *SI Appendix*, Fig. S3*A*), indicating defective follicular development beyond the primary stage. Nevertheless, MTOR was not necessary for the important transition of primordial oocytes to the activated growing oocyte stage. No aspect of follicular development in *Mtor*-ZcKO females was different from controls (*SI Appendix*, Fig. S3*B*). Thus, although MTOR deletion in primordial stage oocytes impacted the development of advancing follicles and oocytes and the ovulation rate, MTOR deletion in growing oocytes did not overtly affect follicular development or ovulation rate, although it did impair the developmental competence of the ovulated oocytes (*SI Appendix*, Table S1).

There was progressive deterioration of follicular development in *Mtor*-GcKO ovaries with aging: At 3 mo, there were fewer normal follicles (*SI Appendix*, Fig. S4*A*), while at 6 mo there were essentially no normal developing follicles (*SI Appendix*, Fig. S4*B*).

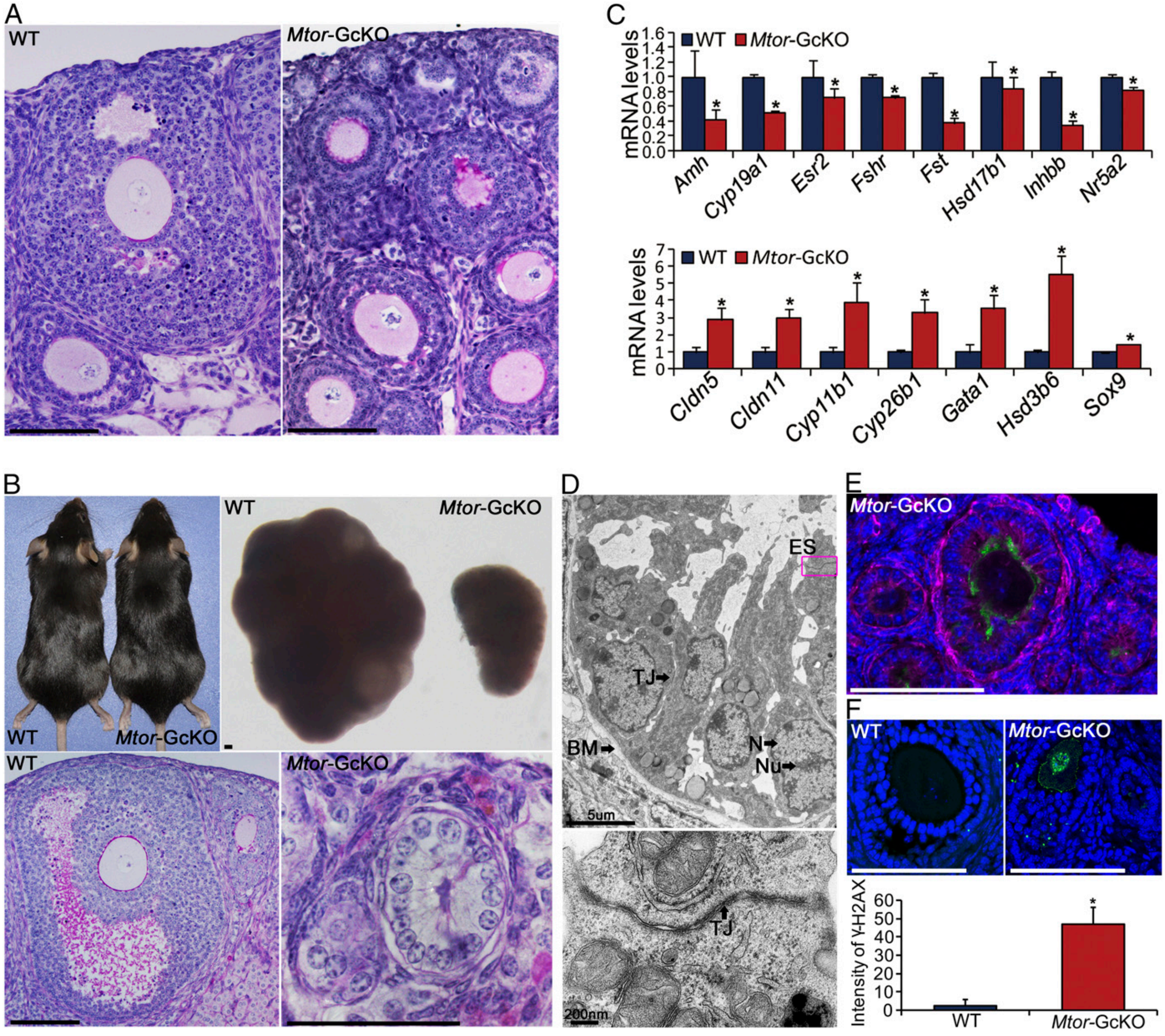

Fig. 2. Defective follicle and granulosa cell development in *Mtor*-GcKO ovaries. (*A*) Micrographs of periodic acid-Schiff (PAS)-stained 21-d-old WT and *Mtor*-GcKO ovarian sections. (*B*, *Upper*) Photographs of whole bodies (*Left*) and ovaries (*Right*) of 6-mo-old WT and *Mtor*-GcKO mice. (*Lower*) Micrographs of PAS-stained ovarian sections of 6-mo-old WT (*Left*) and *Mtor*-GcKO 9 (*Right*) mice. (*C*) qRT-PCR analyzing the expression of a cohort of genes characteristic of ovarian granulosa cells (*Top*) and testicular Sertoli and/or Leydig cells (*Bottom*) in 6-mo-old WT and *Mtor*-GcKO ovaries. (*D*, *Upper*) Transmission electron microscopic imaging of a 6-mo-old *Mtor*-GcKO mouse ovarian follicle with abnormal somatic cells that resemble immature Sertoli-like cells. (*Lower*) Magnified view of the boxed area in the *Upper* image indicated as ectoplasmic specialization (ES). BM, basal membrane; N, nucleus; Nu, nucleolus; TJ, tight junction. (*E*) IF staining of CLDN5 in 6-mo-old *Mtor*-GcKO ovaries. CLDN5, ZP2, and DNA are stained magenta, green, and blue, respectively. (*F*, *Upper*) IF staining of γH2AX in 5-wk-old WT and *Mtor*-GcKO ovaries. γH2AX and DNA are stained green and blue, respectively. (*Lower*) The bar graph shows the quantification of the γH2AX staining. *$P < 0.05$, compared with the WT or control by student's *t* test. Data represent the mean ± SEM. (Scale bars, *A*, *B*, *E*, and *F*, 100 μm.)

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The 6-mo ovaries were smaller and had many abnormal early-stage growing follicles that were surrounded by a prominent basal lamina and were devoid of normal oocytes (Fig. 2 *B* and *D* and *SI Appendix*, Fig. S4*B*). The somatic cells within these abnormal follicles showed some characteristics of immature Sertoli cells rather than granulosa cells. They exhibited veil-like elongated cytoplasm extending toward the center of the follicle and a round nucleus locating near the basal membrane and formed tight junctions with the adjacent partners (Fig. 2 *B* and *D*). There were no tripartite nucleoli characteristic of mature Sertoli cells; rather, the cells appeared similar to immature Sertoli cells of 7-d-old mice (17). No typical tight junctions were found in the WT early-stage growing follicles (*SI Appendix*, Fig. S5*A*). Consistent with the morphological similarity to Sertoli cells, transcriptomic analysis by RNA sequencing (RNA-seq) (*SI Appendix*, Fig. S5*B*) revealed that genes normally expressed by ovarian granulosa cells and essential for granulosa cell development and steroidogenesis, i.e., *Amh*, *Cyp19a1*, *Esr2*, *Fshr*, *Fst*, *Hsd17b1*, *Inhbb*, and *Nr5a2* (18–24), were down-regulated (Fig. 2*C*), while genes characteristic of testicular Sertoli and/or Leydig cells and normally not expressed by granulosa cells, i.e., *Cldn5*, *Cldn11*, *Cyp11b1*, *Cyp26b1*, *Gata1*, *Hsd3b6*, and *Sox9* (25–30), were up-regulated in *Mtor*-GcKO ovaries (Fig. 2*C*). Moreover, CLDN5, an essential component of tight junctions that form the blood–testis barrier in testis, was robustly expressed by these apparently transdifferentiated granulosa cells in a pattern similar to that expressed by Sertoli cells but was barely detected in granulosa cells of WT ovaries (Fig. 2*E* and *SI Appendix*, Fig. S6*A*). Given that no tight junctions have been observed in mouse early-stage growing follicles (31), these data indicate that *Mtor*-GcKO ovarian granulosa cells lost their unique female identity and acquire male Sertoli cell-like characteristics. These Sertoli-like structures were found only when *Mtor* was deleted in primordial, but not in growing, oocytes.

In addition to oocyte loss, reduction of steroidogenic gene expression, and ovarian size, the levels of estradiol and progesterone in circulation were low in 6-mo-old *Mtor*-GcKO females (*SI Appendix*, Fig. S6*B*). In contrast, follicular development in 6-mo-old *Mtor*-ZcKO mice appeared normal (*SI Appendix*, Fig. S4*C*), and steroid hormone levels were not changed (*SI Appendix*, Fig. S6*C*).

What factors contributed to oocyte loss and could give rise to the immature Sertoli cell-like structures prevalent in *Mtor*-GcKO ovaries? A similar transdifferentiation of ovarian cells to Sertoli-like cells occurred after oocyte-lethal irradiation of rats (9). Oocyte death is one consequence of a self-surveillance mechanism to defend genome integrity against the accumulation of excessive DNA damage (32, 33). We therefore assessed potential DNA damage in *Mtor*-GcKO oocytes. IF staining of γH2AX revealed more DNA double-strand breaks (DSBs) in *Mtor*-GcKO oocytes of the early-stage growing follicles (Fig. 2*F*). No increase in γH2AX staining was evident in *Mtor*-ZcKO oocytes at the similar stage (*SI Appendix*, Fig. S4*D*). Hence, *Mtor* deletion in primordial oocytes may result in progressive accumulation of DSBs in developing oocytes and acute loss of these oocytes with age in *Mtor*-GcKO ovaries. While DNA damage may instigate oocyte death that leads to the loss of granulosa identity, there are probably other factors downstream of MTOR deletion that contribute to the phenotype of defective granulosa cell development. Indeed, oocyte death does not necessarily always cause identity loss in its associated granulosa cells. Although irradiation induces oocyte loss in rat primordial follicles and leads to the subsequent transdifferentiation of granulosa cells into Sertoli-like cells in the early-stage growing follicles (9), ablation of mouse oocytes at the similar stages by expressing diphtheria toxin does not result in the same phenotype (11). Despite this conundrum, our observations suggest that a unique MTOR-dependent pathway exists in primordial oocytes that sustains the sex-specific developmental and functional identity of granulosa cells during the later growth stage of these oocytes.

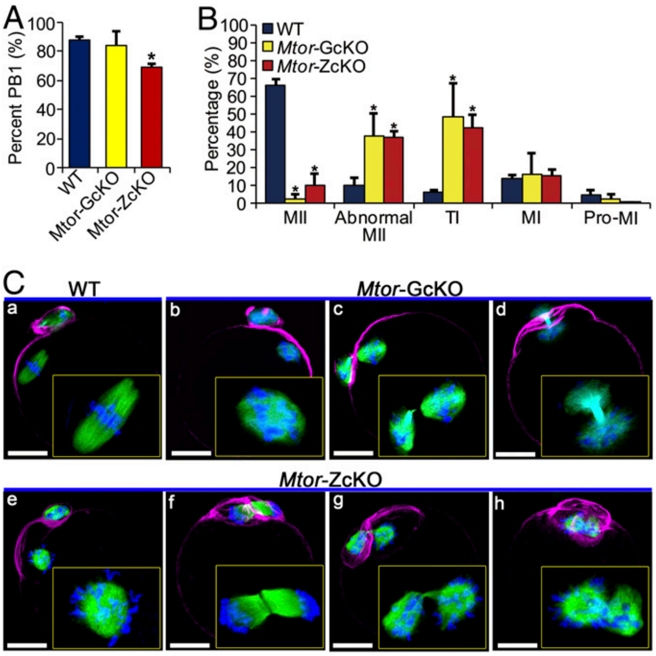

Fig. 3. Defective meiotic progression to MII in ovulated oocytes of both cKO mice. (*A*) First polar body (PB1) rate in ovulated oocytes. (*B*) Quantification of the meiotic stages of ovulated oocytes. TI, telophase I. (*C*) Micrographs showing typical meiotic stages prevalent in WT (*a*), *Mtor*-GcKO (*b*–*d*), and *Mtor*-ZcKO (*e*–*h*) ovulated oocytes. (*a*) Normal MII stage. (*b* and *e*) Abnormal MII with a deformed spindle and misaligned chromosomes. (*c*, *d*, *g*, and *h*) Stages with defective cytokinesis. (*f*) Telophase I. Microtubules, chromosomes, and F-actin are stained green, blue, and magenta, respectively. (Scale bars, 20 μm.) $*P < 0.05$, compared with the WT or control by student's *t* test. Data represent the mean ± SEM.

Oocyte-Specific Knockout of *Mtor* Impairs Completion of the First Meiotic Division in Oocytes. Meiotic errors reduce egg quality (34). Oocyte meiotic progression was therefore determined to assess possible mechanisms for the diminishment of egg quality in cKO females. Even though *Mtor*-cKO mutant oocytes produced the first polar body with nearly the same frequency as WT oocytes (Fig. 3*A*), oocytes ovulated by both cKOs did not complete the first meiosis normally (Fig. 3 *B* and *C*): 78.2% and 65.5% of *Mtor*-GcKO and *Mtor*-ZcKO oocytes, respectively, either formed abnormal metaphase II (MII) spindles (Fig. 3 *C*, *b* and *e*) or did not complete cytokinesis and remained at telophase I (Fig. 3 *C*, *c*, *d*, and *f*–*h*). These defects were recapitulated when cKO oocytes underwent maturation in vitro (*SI Appendix*, Fig. S7), as shown by live imaging of spindles and chromosomes of fluorescent protein-tagged tubulin and histone (*SI Appendix*, Fig. S8). Therefore, the failure of meiotic progression to MII reflects the diminished quality of both cKO oocytes even though the deletion of *Mtor* occurred in much earlier-stage oocytes. These meiotic defects could be far-downstream consequences of the initial MTOR deficiency.

Differential Effect of *Mtor* Deletion at the Primordial and Growing Oocyte Stage on Transcriptomic Integrity of FGOs. Transcriptomic differences in steady-state levels of mRNAs expressed by both cKO FGOs were assessed by RNA-seq analyses. A remarkable difference was observed between the transcriptomes of *Mtor*-GcKO and *Mtor*-ZcKO FGOs: 979 transcripts were expressed differentially between them (Fig. 4*A*). Compared with WT FGOs, the transcriptome was changed more profoundly in *Mtor*-GcKO FGOs, with a significant difference in the expression of 447 transcripts, while in *Mtor*-ZcKO oocytes the changes were relatively minor; only 85 transcripts were differentially expressed

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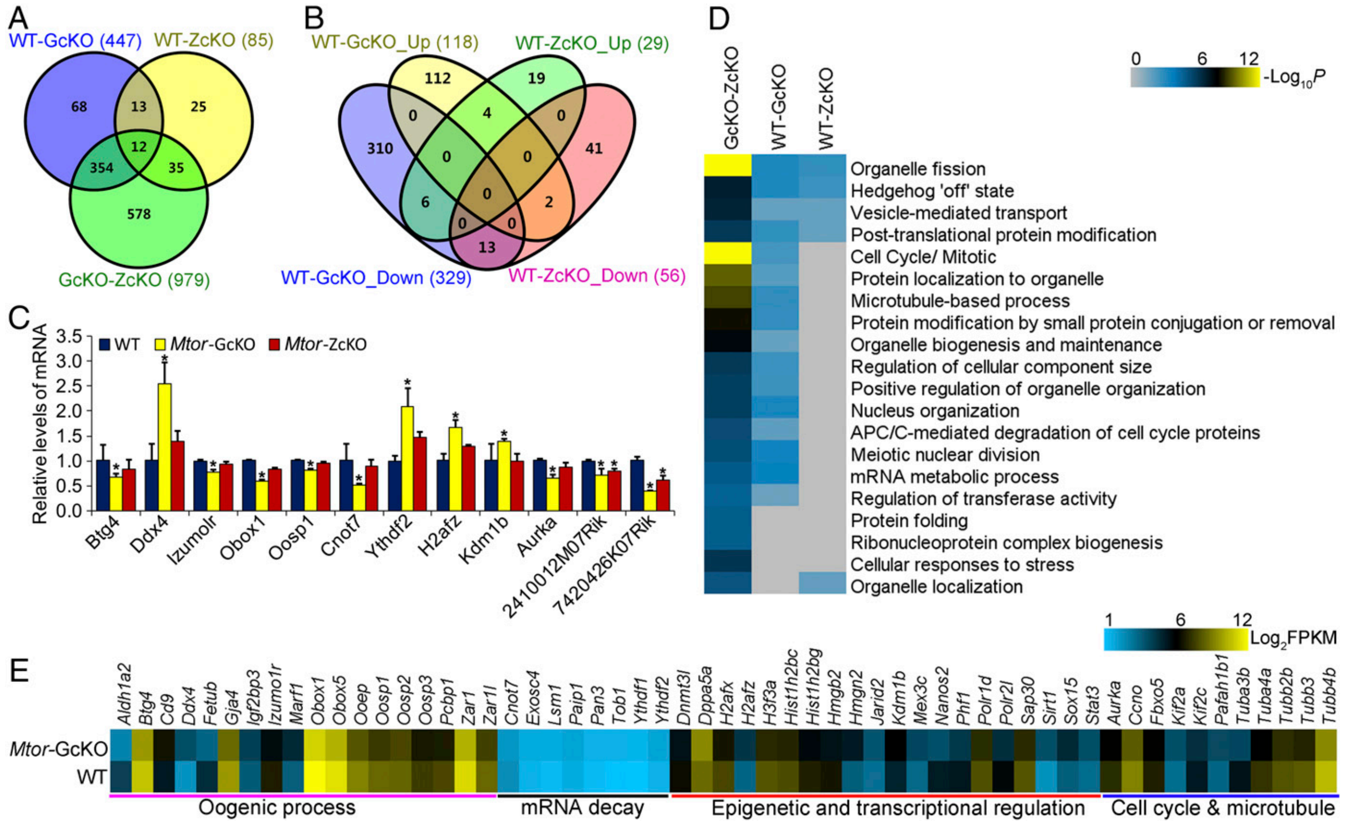

Fig. 4. Differential effect of *Mtor* cKO on the integrity of transcriptome of FGOs. (*A*) Venn diagram illustrating the relationship of the changed transcripts identified by RNA-seq in *Mtor*-GcKO and *Mtor*-ZcKO FGOs. WT–GcKO: WT vs. *Mtor*-GcKO; WT–ZcKO: WT vs. *Mtor*-ZcKO; GcKO–ZcKO: *Mtor*-GcKO vs. *Mtor*-ZcKO. The total number of changed transcripts is indicated in parentheses. (*B*) Venn diagram illustrating the relationship of up- and down-regulated transcripts identified by RNA-seq in *Mtor*-GcKO and *Mtor*-ZcKO FGOs. WT–GcKO_Up: up-regulated in *Mtor*-GcKOs compared with WT; WT–ZcKO_Up: up-regulated in *Mtor*-ZcKOs compared with WT; WT–GcKO_Down: down-regulated in *Mtor*-GcKOs compared with WT; WT–ZcKO_Down: down-regulated in *Mtor*-ZcKOs compared with WT. The number of changed transcripts in each group is shown in parentheses. (*C*) Real-time qRT-PCR validating changes in representative transcripts selected from RNA-seq data. (*D*) Heatmaps illustrating the enriched terms (GO/KEGG terms or canonical pathways) associated significantly with changed transcripts identified by RNA-seq in *Mtor*-GcKO and *Mtor*-ZcKO FGOs. GcKO–ZcKO: *Mtor*-GcKO vs. *Mtor*-ZcKO; WT–GcKO: WT vs. *Mtor*-GcKO; WT–ZcKO: WT vs. *Mtor*-ZcKO. (*E*) Heatmaps illustrating differences between WT and *Mtor*-GcKO FGOs in the expression of a cohort of transcripts involved in various processes.

(Fig. 4*A* and *SI Appendix*, Tables S3 and S4). Moreover, among transcripts changed in either cKO, there were very few that were in common (Fig. 4*B*). Likewise, Gene Ontology (GO) terms associated with changed transcripts had little in common (Fig. 4*D*). The changes in the expression of a group of representative transcripts were validated by real-time RT-PCR (Fig. 4*C*). Interestingly, it has been reported that the transition from primordial to growing oocytes produces the most dramatic changes in oocyte gene expression, both in the level of transcript expression and their diversity (35). Since the immediate effects of MTOR are primarily posttranscriptional (36), the transcriptomic changes reported here reflect downstream consequences of MTOR action with deletion of *Mtor* at the primordial stage having greater transcriptional impact for oocyte and follicular development.

The greater impact of *Mtor*-GcKO on the downstream transcriptome of FGOs paralleled the greater severity of phenotypic differences in oocyte and follicular development. Gene-enrichment analysis revealed some of the transcriptomic changes in *Mtor*-GcKO oocytes that could bring about the observed defects in oocyte and follicular development. These include genes controlling key oogeneic processes, oocyte mRNA decay, epigenetic and transcriptional control, cell cycle, and microtubule-related processes (Fig. 4 *C* and *E*). For example, down-regulation of the expression of genes involved in oocyte–granulosa communication (i.e., *Gja4* and *Oosp1*, *-2*, *-3*) and the development and survival of oocytes (i.e., *Aldh1a2*) in *Mtor*-GcKO oocytes could potentially affect the development and functions of oocyte-companion granulosa cells (37–42). In addition, up-regulation of several genes for mRNA decay could dysregulate the transcript dosage of certain factors key for oocyte and follicle development. These include *Ythdf2* and *Lsm1*, both of which are implicated in the control of oocyte maturation processes and are essential for female fertility (38, 43).

***Mtor* Deletion in Growing Oocytes Alters the Oocyte Proteome.** Meiotic progression in transcriptionally silent FGOs is exquisitely coordinated with selective translation of some maternal transcripts that are synthesized and stored during oocyte growth (44, 45). This coordination is essential for oocyte completion of the first meiotic division and supporting preimplantation development (44, 46). Since MTOR has a crucial role in the control of cellular translation (36), and *Mtor* deletion in growing oocytes has only a minor impact on oocyte transcriptome, the defects of meiotic and developmental competence observed in *Mtor*-ZcKO oocytes could be caused by aberrant oocyte translation during maturation. We therefore compared the protein-expression profile of *Mtor*-ZcKO oocytes with that of WT oocytes by liquid chromatography–mass spectrometry (LC-MS).

About 4,000 ovulated oocytes of each genotype (WT and *Mtor*-ZcKO) were collected and used for proteomic analysis. A

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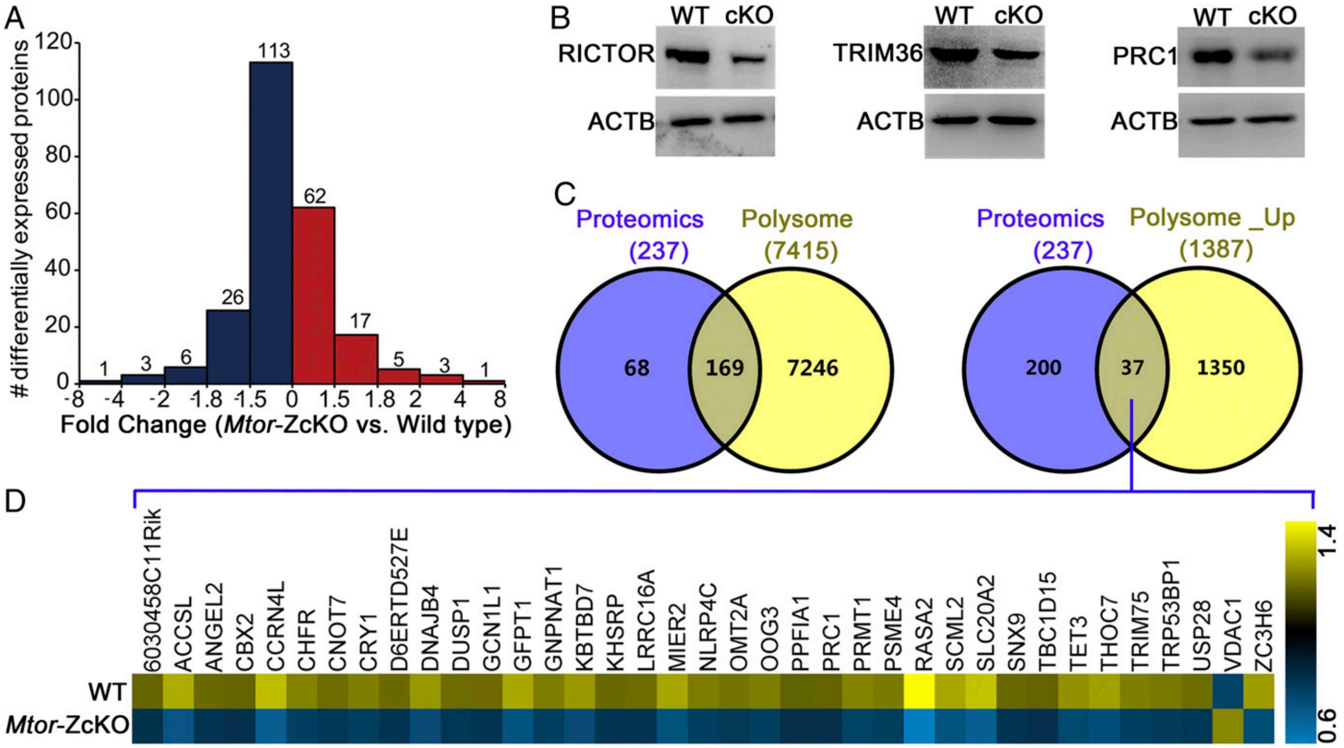

Fig. 5. Proteomic analysis of *Mtor*-ZcKO ovulated oocytes. (*A*) Distribution of significantly changed proteins at various magnitudes of difference in expression levels between *Mtor*-ZcKO and WT ovulated oocytes detected by LC-MS. The number of changed proteins in each category of fold change is indicated above each bar. (*B*) Western blot analysis of RICTOR, TRIM36, PRC1, and ACTB expression in WT and *Mtor*-ZcKO (cKO) ovulated oocytes. (*C*) Venn diagrams illustrating the relationship of the proteomic dataset with polysome RNA array datasets on mRNAs translated in WT FGOs at all stages, i.e., GV, MI, and MII stages, indicated as "Polysome" (*Left*) or translated in oocytes only during the transition from GV to MII, indicated as "Polysome_Up" (*Right*) (44). (*D*) Heatmaps illustrating differences between WT and *Mtor*-ZcKO ovulated oocytes in the expression of proteins that are in common between the Proteomics dataset and the Polysome_up dataset.

total of 4,172 proteins were detected, of which 237 were differentially expressed by *Mtor*-ZcKO (Fig. 5*A* and *SI Appendix*, Table S5). Changes were validated by Western blot analysis of selected representatives, i.e., RICTOR, TRIM36, and PRC1 (protein regulator of cytokinesis 1) (Fig. 5*B*). Gene-enrichment analysis revealed that the 149 down-regulated and the 88 up-regulated proteins participate in different processes (*SI Appendix*, Fig. S9). In particular, the processes "mRNA metabolic process" and "actin filament bundle assembly" are among the GO terms associated with down-regulated proteins. Since selective degradation of maternal transcripts is an important part of the oocyte cytoplasmic maturation process (43, 47, 48), and actin dynamics drive oocyte meiotic division (49, 50), down-regulation of the expression of proteins involved in these two processes is likely detrimental to oocyte maturation and contributes to the defects observed in *Mtor*-ZcKO oocytes.

Deleted in azoospermia-like (DAZL) is reported to drive the translation of a specific subset of maternal mRNAs (e.g., *Tpx2*, targeting protein for *Xenopus* kinesin xklp2) during oocyte meiotic maturation (44). This oocyte maturation-requiring translational program is thought to be mediated by the PI3K–AKT–MTOR pathway (46). Surprisingly, our proteomic analysis did not detect significant changes of either DAZL or TPX2 proteins in ovulated *Mtor*-ZcKO oocytes (*SI Appendix*, Table S5). This suggests that the oocyte meiotic progression-associated translation of DAZL and TPX2 is not dependent on MTOR and that meiotic defects in *Mtor*-ZcKO oocytes were not caused directly by the alteration of DAZL and TPX2 expression. This unexpected observation prompted us to ask which MTOR-dependent proteins are produced during maturation. To address this, we compared the proteins expressed differentially in WT and *Mtor*-ZcKO oocytes with those identified by Chen et al. (44) on polysome-bound mRNA in oocytes during maturation. Accordingly, translation of 169 proteins in germinal vesicle (GV), metaphase I (MI), or MII oocytes was apparently MTOR dependent (Fig. 5*C*, *Left*). Of these, 37 were selectively translated in MII oocytes (Fig. 5*C*, *Right*). Therefore, MTOR signaling in growing oocytes controls the translation of 37 proteins downstream during the GV-to-MII transition in oocytes. Of these, 36 were expressed at lower levels in *Mtor*-ZcKO oocytes compared with WT oocytes (Fig. 5*D*), indicating that their expression was promoted downstream during oocyte maturation by the MTOR pathway expressed during the oocyte growth phase. Identification of these MTOR-controlled proteins provided candidates for further investigation of their role in control of oocyte maturation and preimplantation development.

MTOR-Dependent Expression of PRC1 Is Crucial for Oocyte Completion of the First Meiotic Division. Levels of PRC1 were down-regulated in *Mtor*-ZcKO oocytes (Fig. 5*B*). Given the indispensable role of PRC1 in controlling cytokinesis during somatic cell division (51), we assessed potential effects of PRC1 down-regulation in *Mtor*-ZcKO oocytes on oocyte maturation. PRC1 was distributed throughout the cytoplasm of normal GV-stage oocytes (Fig. 6*A* and *SI Appendix*, Fig. S10) and localized to microtubules around chromosomes at prometaphase I, MI, and MII (Fig. 6*A* and *SI Appendix*, Fig. S10). It was concentrated in the central spindle region at anaphase I and accumulated at midbodies at telophase I (Fig. 6*A* and *SI Appendix*, Fig. S10). To determine if PRC1 functions during oocyte meiotic progression, *Prc1* expression was knocked down in WT oocytes using *Prc1* morpholinos. This led to meiotic defects similar to those observed in ovulated

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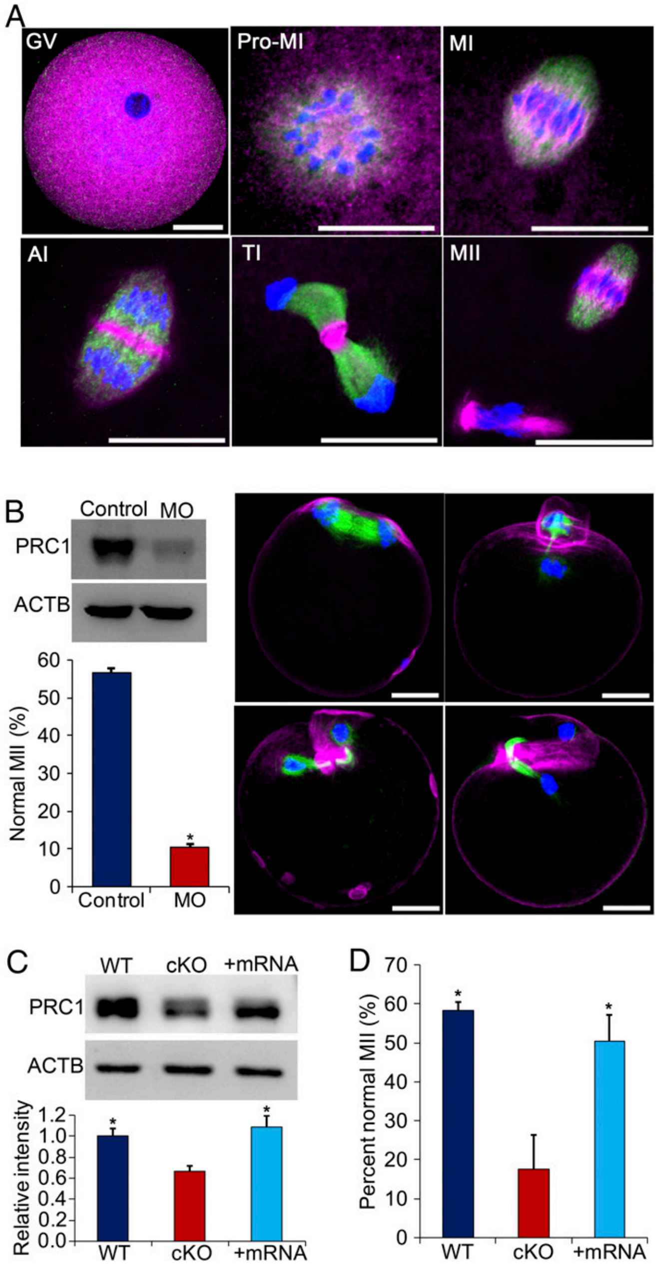

Fig. 6. Localization and function of PRC1 in oocytes during meiotic maturation. (*A*) PRC1 localization in WT oocytes at different meiotic stages. (*B*) Effect of PRC1 knockdown on oocyte meiotic progression in WT cells. (*Upper Left*) Knockdown of PRC1 by *Prc1*-morpholino (MO). (*Lower Left*) The graph shows the percentage of oocytes in which meiosis progressed normally to MII. (*Right*) Micrographs demonstrate typical cytokinesis defects in PRC1–knocked-down oocytes. PRC1/F-actin, microtubules, and chromosomes are stained magenta, green, and blue, respectively. (*C* and *D*) Rescuing cytogenesis defects of *Mtor*-ZcKO oocytes by PRC1 overexpression. *Mtor*-ZcKO GV oocytes were microinjected with *Prc1* mRNA and matured in vitro for 18 h. Meiotic status was then analyzed by IF staining of chromosomes and spindles. (*C*, *Upper*) The Western blot gel image detecting PRC1 expression. (*Lower*) The graph shows the quantification of Western blot results. (*D*) The quantification of oocytes at normal MII stage. “+mRNA” indicates microinjection with *Prc1* mRNA. $^*P < 0.05$, compared with the cKO-group by student’s *t* test. (Scale bars, 20 μm.)

Mtor-ZcKO oocytes (Fig. 6*B*). Very few (~10%) *Prc1* morpholino-injected oocytes reached the MII stage (Fig. 6*B*, *Lower Left*), and most of them displayed defective cytokinesis (Fig. 6*B*, *Right*). Moreover, microinjection of polyadenylated *Prc1* mRNA into *Mtor*-ZcKO oocytes partially rescued their defects in the first meiotic division (Fig. 6 *C* and *D*). Therefore, PRC1 is an MTOR pathway-dependent protein essential for the progression of meiosis to metaphase II in oocytes. cKO of the MTOR pathway in growing oocytes has downstream effects on oocyte maturation and fertility caused, at least in part, by decreasing PRC1 levels.

Conclusion

Using oocyte stage-specific cKOs of *Mtor*, we distinguished the roles of MTOR-dependent pathways in primordial and growing oocytes in controlling downstream oocyte and follicular development and revealed some of the mechanisms by which each causes infertility (*SI Appendix*, Table S1). When MTOR was deleted in primordial oocytes, it was also deleted in the later stages of growing oocytes and FGOs (Fig. 1*A*). Therefore, an overlap in the processes affected in both cKOs would be expected. In fact, meiosis and developmental competence were affected in both. However, other phenotypes, such as aberrant follicular development, transdifferentiation of granulosa cells to immature Sertoli-like cells, and DNA damage, were exhibited in *Mtor*-GcKO, but not in *Mtor*-ZcKO, ovaries. Thus, additional processes affected in *Mtor*-GcKO ovaries are attributable to MTOR-dependent pathways having different functional impacts in primordial oocytes and in growing oocytes, even though these phenotypes were not manifest until later in oocyte and follicular development. Once oocyte growth begins, MTOR promotes the completion of the first meiotic division and preimplantation embryogenesis and no longer appears to be involved in oocyte genomic protection, granulosa cell fate determination, or follicular development.

Materials and Methods

Mice. *Mtor*-floxed and *Gdf9*-, *Zp3*-Cre mice were obtained from The Jackson Laboratory and were maintained on identical C57BL/6J genetic backgrounds. The procedures of mouse breeding, genotyping, and fertility testing are detailed in *SI Appendix*. All mouse procedures and protocols were approved by the Animal Care and Use Committee at Nanjing Medical University and were conducted in accordance with the institutional guides for the care and use of laboratory animals.

Chemicals and Reagents. Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich Co.

Hormone Assays. Sera were collected as described previously (52), and hormones in them were measured using the methods detailed in *SI Appendix*.

Histology, Immunohistochemistry, and Western Blot Analysis. These analyses and follicle count were carried out as described previously (15, 53–56) and are detailed, along with information about the antibodies used, in *SI Appendix*.

Oocyte Isolation, in Vitro Manipulation, and Imaging. These were carried out as described previously (15, 56) and are detailed in *SI Appendix*.

Proteomic, RNA-Seq, and qRT-PCR Analyses. About 4,000 WT and *Mtor*-ZcKO ovulated oocytes were collected for the proteomic analysis. Transcriptomic analyses were carried out using RNA-seq. Real-time qPCR analysis was carried out as described previously (48). Detailed procedures are described in *SI Appendix*. RNA-seq data have been deposited in the Gene Expression Omnibus (datasets GSE98497 and GSE98548), and proteomics data have been deposited in the ProteomeXchange Consortium (dataset PXD006408).

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