

Murine Follicular Development Requires Oocyte DICER, but Not DROSHA¹

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ABSTRACT

Both DICER and DROSHA are RNase III enzymes involved in the biogenesis of small noncoding RNAs. DROSHA cleaves the stem-loop portion of the primary miRNAs and produces precursor miRNAs in the nucleus, whereas DICER processes double-stranded RNA precursors into mature miRNAs and endogenous small interference RNAs in the cytoplasm. Selective inactivation of *Dicer* in growing oocytes of primary follicles leads to female infertility due to oocyte spindle defects. However, it remains unknown if oocyte *Dicer* expression in the fetal ovary is required for proper follicular development in the postnatal ovary. Moreover, the role of *Drosha* in folliculogenesis has never been investigated. Here, we report that conditional knockout of *Dicer* in prophase I oocytes of the fetal ovary led to compromised folliculogenesis, premature ovarian failure, and female infertility in the adult ovary, whereas selective inactivation of *Drosha* in oocytes of either the fetal or the developing ovary had no effects on normal folliculogenesis and female fertility in adulthood. Our data indicate that oocyte DICER expression in the fetal ovary is required, and oocyte DROSHA is dispensable, for postnatal follicular development and female fertility in adulthood.

fertility, meiosis, oocyte, oogenesis, ovary, ovulation, posttranscriptional regulation, premature ovarian failure, primordial germ cells, small RNA, spindle

INTRODUCTION

Sexual reproduction requires gametes from both sexes. Male and female gametes (i.e., sperm and oocytes) are both derived from primordial germ cells (PGCs) [1]. During embryonic development, both male and female PGCs multiply themselves through mitosis and then adopt different differentiation pathways: prospermatogonia in the testis remain in G₀ phase until the onset of pubertal testicular development, whereas oogonia in the ovary enter meiosis prophase I and become oocytes followed by a late prophase I arrest at birth [2, 3]. The number of available primordial follicles at birth, which is believed to represent the total follicular reserve for the entire reproductive lifespan in a female, is determined by the proliferation rate of the female PGCs and also the rate of PGCs committing meiosis during embryonic ovarian development [4]. Upon puberty, mature oocytes are produced via

folliculogenesis, a process through which primordial follicles develop into primary, secondary, and eventually antral follicles competent for ovulation. During folliculogenesis, both oocytes and follicular cells are subject to complex regulation of gene expression, which can occur at multiple levels, including transcriptional, posttranscriptional, and posttranslational [5]. In oocytes, posttranscriptional regulation is prominent because numerous maternal transcripts need to be produced and stored for usage during fertilization and during postfertilization development [6]. RNA-binding proteins (RBPs) have long been suggested to be involved in the control of mRNA fate, and thus research efforts have been focused on oocyte-expressed RBPs for the past several decades [6]. Small noncoding RNAs (sncRNAs) represent another critical component in the complex machinery of posttranscriptional regulation [7–9]. Among numerous sncRNAs, miRNAs and endogenous small interference RNAs (endo-siRNAs) are well studied and both function to affect stability and translational efficiency through binding 3' untranslated regions of their target mRNAs [7, 10]. The biogenetic pathways of these two types of sncRNAs have been defined, both of which involve a cytoplasmic RNase III, DICER, and miRNA production also requires another nuclear RNase III, i.e., DROSHA [11–14].

Similar to mRNA genes, miRNA genes are transcribed into primary miRNA transcripts (pri-miRNAs) with 5' caps, 3' polyA tails, and a stem-loop region containing the mature miRNA sequences [15]. The stem-loop portion of pri-miRNAs can be recognized by the microprocessor complex, which contains DROSHA, a nuclear RNase III, and its cofactor DGCR8, and cleaves to form precursor miRNAs (pre-miRNAs), which are then exported from the nucleus to the cytoplasm by a RAN-binding protein called EXPORTIN5 [16, 17]. Once in the cytoplasm, DICER recognizes the double-stranded RNA in the stem-loop region of pre-miRNAs and cleaves out two mature miRNAs [15]. Endo-siRNAs are processed by DICER in the cytoplasm using naturally formed double-stranded RNAs (dsRNAs), which can be derived from two mRNAs with partial segments that are complementary [15]. Endo-siRNAs have been identified in male and female germ cells as well as in embryonic stem cells [15]. Because endo-siRNAs are processed in the cytoplasm, its biogenesis requires DICER, but not DROSHA [15–17].

The early embryonic lethality phenotype (i.e., embryo dies at Embryonic Day 6.5 [E6.5]–E7.5) in global *Dicer*, *Dgcr8*, or *Drosha* knockout (KO) mice highlights the necessity of miRNAs and endo-siRNAs in development [11–14]. Studies using *Dicer* or *Drosha* conditional KO (cKO) mice have demonstrated that miRNAs and endo-siRNAs play critical roles in both the development and functions of almost all organs and cell types [18]. *Dicer* plays an essential role in both male and female germ cell development [19–27]. In the male, selective inactivation of *Dicer* or *Drosha* in postnatal spermatogenic cells causes disrupted spermatogenesis characterized by severe depletion of spermatocytes and spermatids, azoospermia, and complete male infertility [21, 23–27]. Although selective inactivation of *Dicer* in growing oocytes

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of primary follicles does not cause obvious disruptions in folliculogenesis, the *Dicer*-deficient oocytes fail to mature properly and display aberrant spindle integrity and misalignment of chromosomes [20]. Consequently, those *Zp3-Dicer* cKO females are completely infertile. This finding was initially interpreted to reflect the necessity of miRNAs in oocyte maturation, but later noted as an effect of endo-siRNA depletion due to DICER deficiency because miRNA depletion by *Dgcr8* inactivation in growing oocytes of primary follicles does not disrupt folliculogenesis, and *Dgcr8*-null oocytes develop normally and are fertile [28]. These earlier studies suggest that it is endo-siRNAs, but not miRNAs, that are essential for oocyte maturation and thus female fertility [29]. In this study, we aimed to answer the following questions: 1) Does *Dgcr8* inactivation phenocopy *Drosha* inactivation? 2) Is *Dicer* or *Drosha* expression in early prophase I oocytes needed for follicular development in the developing and adult ovaries? 3) Is DROSHA required for folliculogenesis and female fertility? Here, we report that inactivation of oocyte *Dicer* in the fetal ovary led to compromised folliculogenesis and premature ovarian failure in the adult ovary, whereas inactivation of *Drosha* in oocytes of either fetal or developing ovaries did not affect folliculogenesis and female fertility in adulthood.

MATERIALS AND METHODS

Animal Use and Generation of cKO Mice

The Institutional Animal Care and Use Committee of the University of Nevada, Reno, approved all animal use protocols. All mice were housed and maintained under specific pathogen-free conditions with a temperature- and humidity-controlled animal facility in the University of Nevada, Reno. All flox and Cre lines used in this study were purchased from the Jackson Laboratory. All mouse lines were backcrossed for five generations to get onto the C57B6/6J background. Adult *Dicer^{flox/flox}* female mice [30] were bred with adult *Ddx4-Cre* male mice [31] to generate *Ddx4-Cre; Dicer^{+flox}* offspring, which were further crossed with *Dicer^{flox/flox}* mice to obtain *Ddx4-Cre; Dicer^{flox/flox}* female mice. *Drosha^{flox/flox}* male mice [14] were bred with *Zp3-Cre* female mice [32] to generate *Zp3-Cre; Drosha^{+flox}* mice, which were then further crossed with *Drosha^{flox/flox}* mice to obtain *Zp3-Cre; Drosha^{flox/flox}* females for this study. To generate *Drosha* global KO mice, *Zp3-Cre; Drosha^{+flox}* female mice were mated with wild-type (WT) male mice to obtain *Drosha^{+del}* mice, which were then further intercrossed to generate *Drosha^{del/del}* (global KO) mice.

Fertility Test and Oocyte Collection

For fertility test, six 6-wk-old *Ddx4-Cre; Dicer^{flox/flox}* or *Zp3-Cre; Drosha^{flox/flox}* female mice were bred with adult WT males of proven fertility for a period of 10 wk. To collect fully grown, germinal vesicle (GV)-intact, cumulus-enclosed oocytes, 4- to 6-wk-old WT, *Ddx4-Cre; Dicer^{flox/flox}*, and *Zp3-Cre; Drosha^{flox/flox}* female mice were primed with equine chorionic gonadotropin (eCG; 5 IU/mouse) via i.p. injection. The primed mice were killed 46–48 h after eCG treatment, and the ovaries were dissected in M2 medium to release GV-stage oocytes. For mature (metaphase II [MII] stage) oocytes, mice were first injected with eCG (5 IU/mouse, i.p.) and subsequently with human chorionic gonadotropin (hCG; 5 IU/mouse, i.p.) 48 h after eCG treatment, followed by collecting MII-stage oocytes with cumulus cells from oviduct 14–16 h after hCG treatment. The GV- and MII-stage oocytes were treated with 0.1% bovine testicular hyaluronidase in M2 medium at 37°C for 2–3 min to remove cumulus cells. The cumulus-free GV and MII oocytes were washed three times in M2 medium and used for subsequent molecular analyses.

Mouse Genotyping

Mouse DNA was prepared by digestion of tail snips in a lysis buffer (40 mM NaOH, 0.2 mM EDTA) for 2 h at 95°C, followed by neutralization using the same volume of neutralizing buffer (40 mM Tris-HCl). PCR reactions were conducted in a 20- μ l volume with tail DNA (1 μ l), H₂O (7 μ l), 2 \times GoTaqGreen master mix (10 μ l) (Promega, Cat. No. M7122), and 10 μ M of forward and reverse primer (1 μ l each). The primers used for genotyping are listed in Table 1. The PCR conditions were 95°C 2 min; (95°C 30 sec, 60°C 30 sec, 72°C 1 min) \times 33 cycles; 72°C 5 min.

Dissection of Early Mouse Embryos

The presence of vaginal plugs in female *Drosha^{+del}* mice mated with *Drosha^{+del}* males was marked as E0.5. At E6.5, E7.5, and E8.5, the pregnant females were euthanized and the embryos were dissected as described [33]. After photography, a portion of the embryo was washed three times in PBS, and then put into individual PCR tubes, each containing 30 μ l of embryo lysis buffer (1 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM MgCl₂, 0.45% NP-40, 0.45% Tween20, and 180 μ g/ml protease K), followed by incubation at 55°C for 5 h and boiling 10 min at 95°C. An aliquot of 5 μ l embryo lysates was used for PCR genotyping using primer sets described in Table 1.

Histology and Immunohistochemistry

Ovaries were dissected and fixed in Bouin fixative overnight at 4°C followed by paraffin embedding. Paraffin sections (5 μ m) were stained with hematoxylin and eosin (H&E) for histological examination. For oocyte immunostaining, oocytes of different genotypes were fixed in 4% paraformaldehyde in M2 medium for 1 h and washed three times in 0.1 M glycine with 0.3 mg/ml bovine serum albumin (BSA) at room temperature (RT), followed by permeabilization with 0.2% Triton X-100 in PBS for 15 min and blocking with a buffer (2% BSA in PBS) for 1 h at RT. The oocytes were then incubated with the following antibodies diluted in blocking buffer for 1 h at RT: rabbit anti-DROSHA polyclonal antibody (Cat. No. ab12286; 1:500 dilution; Abcam) and mouse anti-beta-tubulin (Cat. No. T5293; 1:500 dilution; Sigma). After three washes with the blocking buffer, oocytes were incubated with fluorescence-conjugated, species-specific secondary antibodies (Alexa Fluor 594 goat anti-mouse IgG [H + L] and Alexa Fluor 568 goat anti-rabbit IgG [H + L]; 1:2000 dilution; Invitrogen Molecular Probes) for 1 h at RT. Finally, the oocytes were counterstained with 4', 6-diamidino-2-phenylindole dilactate (DAPI; Sigma) for indirect immunofluorescent assays using a fluorescence microscope (AxioVision; Carl Zeiss).

Quantitative Analyses of Follicles

Quantitative analyses of various follicles in cKO and WT control ovaries at Postnatal Day 30 (P30), P40, and P120 were conducted as described [34]. Briefly, serial sections (8 μ m) of the ovarian paraffin blocks were cut, and every 10th section was mounted onto slides, followed by H&E staining. The numbers of primordial, primary, secondary, and antral follicles were then counted under a microscope. To avoid counting follicles twice, only follicles with a visible nucleolus in the oocyte were counted. The number of follicles on each slide was multiplied by 10 and subsequently multiplied by 8 to obtain the estimated total number of follicles in each ovary.

Single-Oocyte Quantitative PCR Analyses

Single GV or MII oocytes were collected from WT, *Ddx4-Dicer* cKO, and *Zp3-Drosha* cKO females and treated with hyaluronidase to remove cumulus cells. Single oocytes were then individually put into PCR tubes containing 5 μ l CellDirect 2 \times Reaction Mix lysis buffer (One-Step quantitative PCR [qPCR] kit; Invitrogen) and immediately frozen in liquid nitrogen and stored at –80°C. Complementary DNA synthesis and specific target amplification using single oocytes were performed according to the manufacturer's instructions (One-Step qPCR kit). The qPCR analyses were conducted using *Drosha*- or *Dicer*-specific primers, and *Gapdh* was used as a reference gene (Table 1).

Statistical Analyses

Data are presented as mean \pm SEM, and statistical differences between datasets were assessed by one-way ANOVA or *t*-test using SPSS 16.0 software. $P < 0.05$ and $P < 0.01$ were considered as significant and highly significant differences, respectively.

RESULTS

Global Inactivation of Drosha Leads to Embryonic Lethality

Global ablation of DGCR8, a cofactor of DROSHA in miRNA biogenesis, led to embryonic lethality at E6.5 [12, 13]. Because DGCR8 acts as an essential cofactor for DROSHA RNase III activity during pre-miRNA production [15, 29], *Drosha* global KO mice should, in theory, phenocopy DGCR8 KOs. Indeed, among 61 pups produced in eight litters by four heterozygous breeding pairs (*Drosha^{+del}* \times *Drosha^{+del}*), only

TABLE 1. Sequences of primers used in this study.

Name	Forward (5' → 3')	Reverse (5' → 3')	Usage
Zp3-Cre	GCGGTCTGGCAGTAAAACTATC	GTGAAACAGCATTGCTGTCACTT	Genotyping
Ddx4-Cre	CACGTGCAGCCGTTTAAGCCGCGT	TTCCATTCTAAACAACACCCTGAA	Genotyping
Dicer ^{flox}	CCTGACAGTGACGGTCCAAAG	CATGACTCTTCAACTCAAAC	Genotyping
Dicer ^{del}	CCTGACAGTGACGGTCCAAAG	CCTGAGCAAGGCAAGTCATTC	Genotyping
Drosha ^{flox}	GCAGAAAGTCTCCCACTCCTAACC	CCAGGGGAAATTAACGAGACTCC	Genotyping
Drosha ^{del}	GCAGAAAGTCTCCCACTCCTAACC	GCTCTCTTCTGGTGTCTGAAGACAAC	Genotyping
Dicer	TTTACACACGCCTCCTACCA	CAGAGGGCAAGTCAGTCAAG	qPCR
Drosha-Exon9	AGACGGTGATCCGATAGTGG	GACTCCGAGCTGTCCATCT	qPCR
Drosha-Exon20	GGACCATCACGAAGGACACT	CACGGGTCTCTGGTTTTGT	qPCR
Gapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA	qPCR

WT (19/61, 31%) and heterozygotes (42/61, 69%) were obtained, and no homozygous pups were ever found, suggesting a potential embryonic lethality phenotype. To determine the timing of embryonic lethality, we collected the embryos from *Drosha* heterozygous females mated with heterozygous males at E6.5, E7.5, and E8.5 and examined the morphology (Fig. 1). *Drosha*^{-/-} embryos were much smaller than WT or heterozygous embryos at E6.5 and degenerated between E7.5 and E8.5 (Fig. 1), suggesting that *Drosha*-null embryos also die at ~E6.5.

Generation of Ddx4-Dicer cKO and Zp3-Drosha cKO Mice

A previous study has demonstrated that conditional inactivation of *Dicer* in growing oocytes of developing follicles causes spindle defects and female infertility in mice [20]. However, it remains unknown whether inactivation of *Dicer* in oocytes of fetal ovaries will affect follicular development in postnatal ovaries. To address this question, we crossed *Dicer*^{flox/flox} female mice with male *Ddx4-Cre* mice. The *Dicer* conditional allele contains two *loxP* sites flanking exon 23 of the *Dicer* gene, which encodes for a critical portion of the catalytic domain [30], whereas the *Ddx4-Cre* line expresses Cre recombinase exclusively in PGCs starting at E15.5 (Fig. 2A) [31]. Thus, the *Ddx4-Cre; Dicer*^{flox/flox} female mice should lack DICER in oocytes of fetal ovaries at E15.5 and thereafter. We also crossed *Drosha*^{flox/flox} mice with a *Zp3-Cre* deleter line, which starts to express Cre exclusively in growing oocytes of primary follicles (Fig. 2A) [32, 35, 36]. The *Drosha* conditional allele has two *loxP* sites flanking exon 9, and the deletion of exon 9 leads to frame shift and multiple premature stop codons [14].

To confirm the inactivation of *Dicer* or *Drosha*, we performed qPCR analyses using oocytes collected from *Ddx4-Cre; Dicer*^{flox/flox} (herein called *Ddx4-Dicer* cKO) and *Zp3-Cre; Drosha*^{flox/flox} (herein called *Zp3-Drosha* cKO) female mice. In *Ddx4-Dicer* cKO oocytes, no expression of exon 23-containing *Dicer* mRNAs was detected, whereas *Drosha* mRNA levels appeared to be slightly reduced (Fig. 2B). Similarly, exon 9-carrying *Drosha* transcripts were completely absent, whereas the exon 20 transcripts persisted despite drastically decreased levels in both GV and MII oocytes collected from *Zp3-Drosha* cKO females (Fig. 2, C and D). Further immunofluorescent staining detected markedly reduced levels of DROSHA in *Drosha* cKO GV-stage oocytes (Fig. 2, E and F). These results indicate that although *Drosha* mRNAs were still transcribed after the loss of exon 9, production of the truncated DROSHA appeared to be minimal. Therefore, *Dicer* and *Drosha* were indeed inactivated in the *Ddx4-Dicer* and *Zp3-Drosha* cKO oocytes, respectively.

Inactivation of Oocyte Dicer in the Fetal Ovary Leads to Compromised Folliculogenesis and Premature Ovarian Failure in the Adult Ovary

Ddx4-Dicer cKO female mice produced no offspring during a 10-wk fertility test by breeding with males of proven fertility, suggesting that *Ddx4-Dicer* cKO females are infertile. To explore potential ovarian defects, we examined the histology of *Ddx4-Dicer* cKO ovaries at P30, P40, and P120. At P30 and P40, WT ovaries contained all types of follicles, including primordial, primary, secondary, and antral follicles, whereas primordial and antral follicles were rarely seen in *Ddx4-Dicer* cKO ovaries (Fig. 3A). At P120, WT ovaries displayed dynamic folliculogenesis with all types of follicles and corpora lutea present, whereas *Ddx4-Dicer* cKO ovaries appeared to lack any developing follicles (Fig. 3A), suggesting a progressive loss of follicles.

To determine the process of follicle depletion in *Ddx4-Dicer* cKO ovaries, we counted various follicles in WT and *Ddx4-Dicer* cKO ovaries at P30, P40, and P120 (Fig. 3, B–E). The number of primordial follicles was significantly smaller in *Ddx4-Dicer* cKO ovaries than in WT ovaries at all three age groups (Fig. 3B). Despite a slight increase in the number of primary follicles in *Ddx4-Dicer* cKO ovaries from P30 to P40, the primary follicle number was much smaller than that in WT ovaries at all three time points (Fig. 3C). Interestingly, although the number of secondary follicles was drastically decreased between P40 and P120 in *Ddx4-Dicer* cKO ovaries, WT and *Ddx4-Dicer* cKO ovaries appeared to contain comparable numbers of secondary follicles at both P30 and P40 (Fig. 3D). Similar to primary follicles, antral follicles were significantly fewer in *Ddx4-Dicer* cKO ovaries than in WT ovaries at all three time points (Fig. 3E). Overall, these results suggest that premature ovarian failure in *Ddx4-Dicer* cKO females most likely results from defects at multiple steps during follicular development, including the reduced primordial follicle formation and enhanced depletion of developing follicles during folliculogenesis. Consistent with the histological observation, superovulation experiments indicated that the numbers of oocytes recovered from *Ddx4-Dicer* cKO mice were only ~50% and ~10% of those from WT females at P40 and P120, respectively (Fig. 3F). These data suggest that folliculogenesis in *Ddx4-Dicer* cKO females is compromised, and the primordial follicle pool is exhausted by the age of ~4 mo, resembling premature ovarian failure in humans.

Earlier studies have shown that although folliculogenesis appears to be normal in *Zp3-Cre; Dicer*^{flox/flox} females, oocytes from these mice display spindle defects [20]. To determine whether oocytes collected from *Ddx4-Dicer* cKO females also had similar defects, we performed immunofluorescent staining of the spindle using β -tubulin antibodies (Fig. 3G). Consistent with the previous report [20], oocytes of *Ddx4-Dicer* cKO

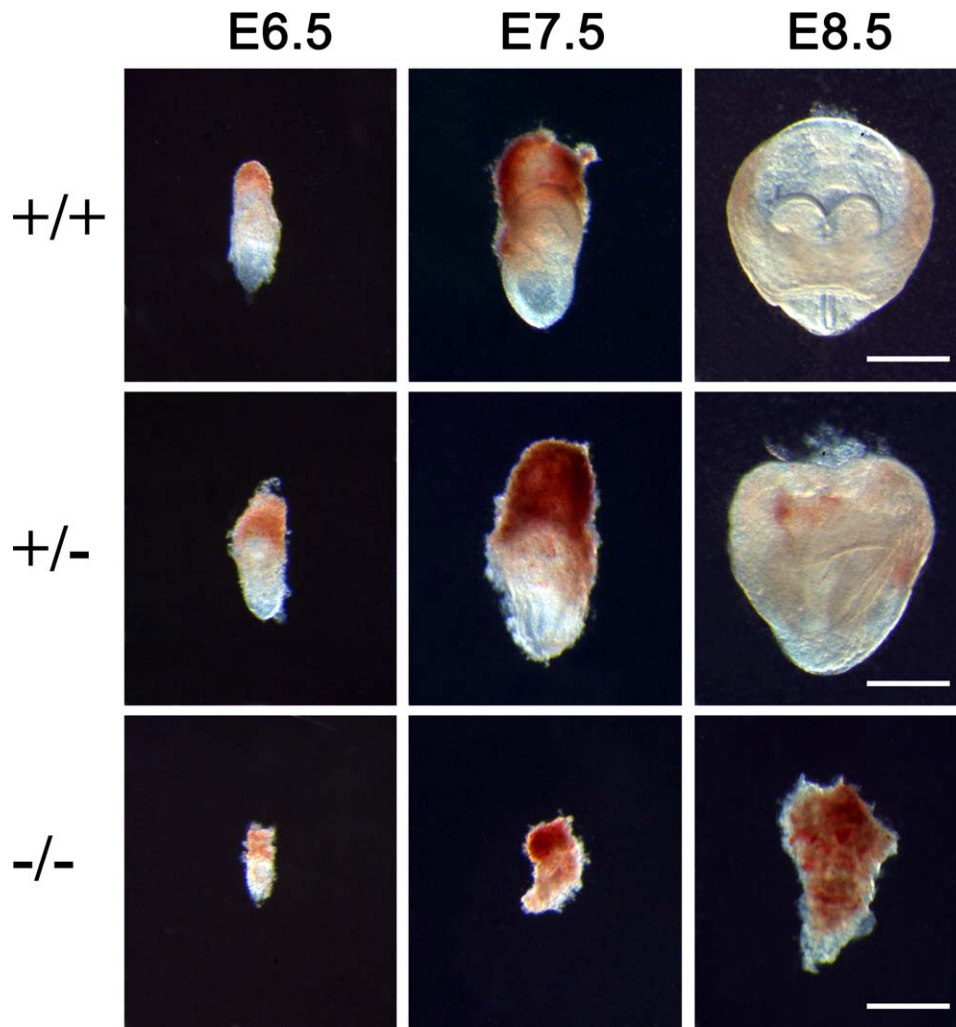


FIG. 1. *Drosha*^{-/-} embryos died at E6.5. WT (+/+) and *Drosha* heterozygous (+/-) embryos developed normally between E6.5 and E8.5, whereas *Drosha*-null (-/-) embryos ceased growth at E6.5 and degenerated by E8.5. Bar = 200 μ m.

females frequently displayed multiple spindles with misaligned chromosomes strewn around the spindle axes, whereas WT oocytes formed a single barrel-shaped spindle with integrated and congregated chromosomes at the metaphase plate (Fig. 3G). Taken together, our data suggest that loss of oocyte *Dicer* in the fetal ovary leads to defective follicular development in the postnatal and adult ovaries.

Oocyte *Drosha* Is Dispensable for Follicular Development

Histology of *Zp3-Drosha* cKO ovaries was indistinguishable from that of WT controls at P40 and P120 (Fig. 4A). Similar numbers of oocytes were recovered from both *Zp3-Drosha* cKO and WT females (Fig. 4B). A 10-wk fertility test by breeding the *Zp3-Drosha* cKO females with fertility-proven WT male mice revealed normal fertility (Fig. 4C). Similar to *Zp3-Drosha* cKO female mice, *Ddx4-Cre; Drosha*^{flox/flox} female mice that we generated were completely fertile (data not shown). Moreover, our breeding records showed no fertility decrease in either *Ddx4-Drosha* cKO or *Zp3-Drosha* cKO female mice older than 6 mo, suggesting that *Drosha* deficiency does not cause latent phenotype in aging female mice. Together, these data suggest that oocyte *Drosha* is dispensable for normal follicular development.

DISCUSSION

DICER is required for the production of both miRNAs and endo-siRNAs, whereas DROSHA is essential for miRNA biogenesis, but dispensable for endo-siRNA production [15, 29]. Several reports have suggested that both DICER and DROSHA may have yet-to-be-defined, snRNA-independent roles [16, 37, 38]. Although miRNA and/or endo-siRNA deficiency is most likely the cause for the phenotype observed in *Ddx4-Dicer* cKO females, disruptions of other DICER-dependent cellular functions couldn't be excluded. We therefore confined this report to phenotypic characterization without snRNA transcriptome analyses.

The DGCR8-DROSHA complex functions as a microprocessor required for pre-miRNA production during miRNA biogenesis [15, 29]. The function was initially established based on data mostly from in vitro experiments using cultured cell lines [38]. If DGCR8 and DROSHA are equally important for pre-miRNA production in vivo, then *Drosha* global KO females should phenocopy *Dgcr8* global KO females. *Dgcr8* global ablation leads to embryonic lethality at E6.5 and cKO of *Dgcr8* in growing oocytes of developing follicles abolishes most of the miRNA production in oocytes, but causes no discernible phenotype [12]. Interestingly, *Drosha* global KO mice also die at E6.5. Similar to *Zp3-Dgcr8* cKO females [12],

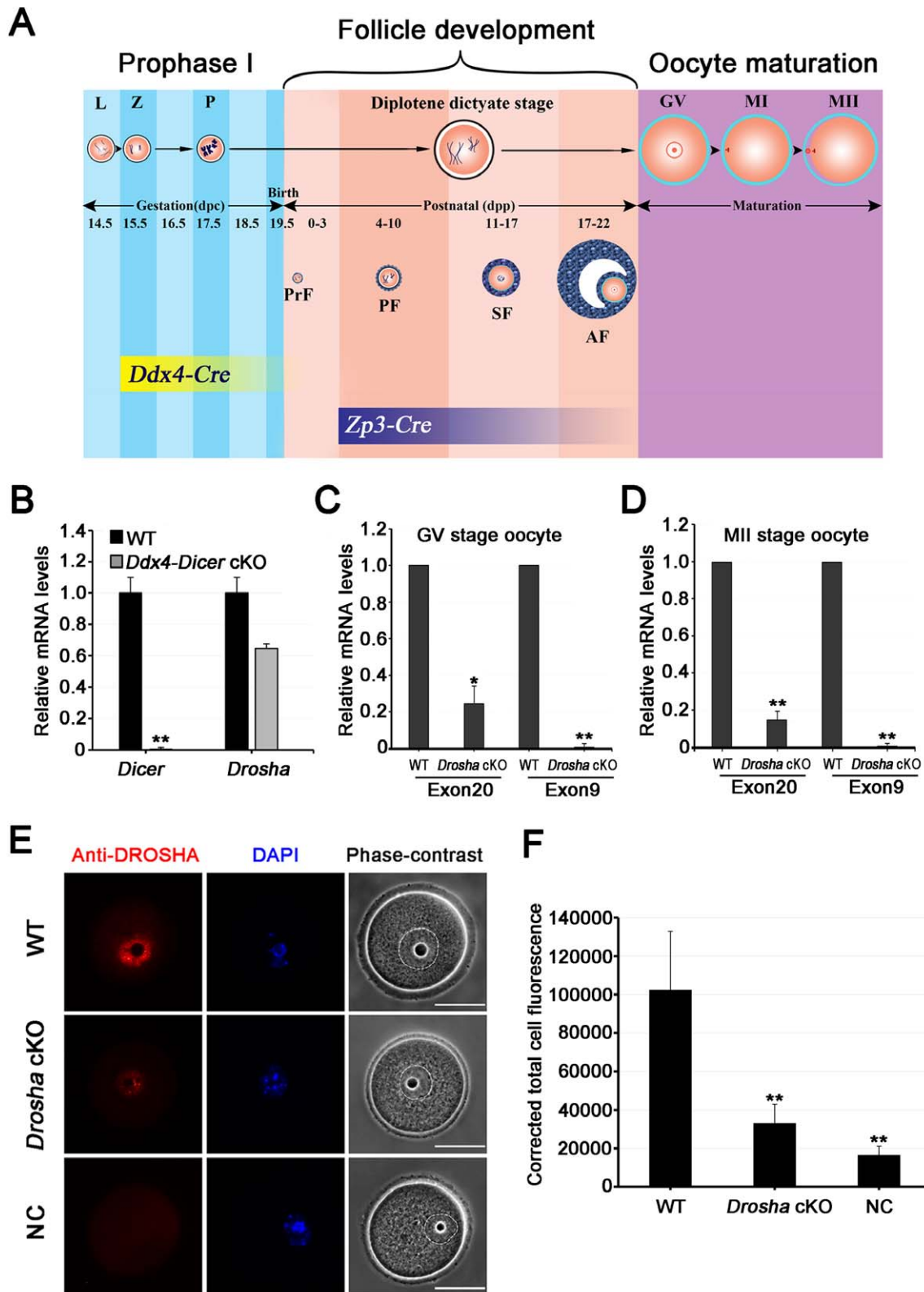


FIG. 2. Selective inactivation of *Dicer* or *Drosha* in prophase I oocytes of the fetal ovary or in growing oocytes of developing follicles. **A**) Schematic timeline of Cre expression during oocyte and follicular development. The *Ddx4-Cre* deleter line starts to express CRE in early prophase I oocytes at E15.5, whereas *Zp3-Cre* mice display CRE activity in growing oocytes of developing follicles. L, leptotene; Z, zygotene; P, pachytene; MI, metaphase I; PrF, primordial follicles; PF, primary follicle; SF, secondary follicle; AF, antral follicle. **B**) The qPCR analyses of *Dicer* and *Drosha* mRNA levels in WT and *Ddx4-Dicer* cKO oocytes. **C**) The qPCR analyses of levels of exon 9- and exon 20-containing *Drosha* mRNAs in WT and *Zp3-Drosha* cKO GV oocytes. **D**) The qPCR analyses of levels of exon 9- and exon 20-containing *Drosha* mRNAs in WT and *Zp3-Drosha* cKO MII oocytes. Note that in all qPCR analyses (**B–D**), relative mRNA levels were determined using the $\Delta\Delta\text{CT}$ method. Data are presented as means \pm SEM ($n = 6$). * $P < 0.05$; ** $P < 0.01$. **E**) Immunofluorescent staining of DROSHA in WT and *Zp3-Drosha* cKO GV-stage oocytes. DNA was visualized with DAPI (blue), and morphology was shown by the phase-contrast images. Dashed lines circle the nuclear membrane. All images were taken using the same exposure time. Bar = 50 μm . **F**) Quantitative analyses of immunofluorescent intensity of DROSHA in WT and *Zp3-Drosha* cKO GV-stage oocytes. Data are presented as means \pm SEM ($n = 10$). ** $P < 0.01$.

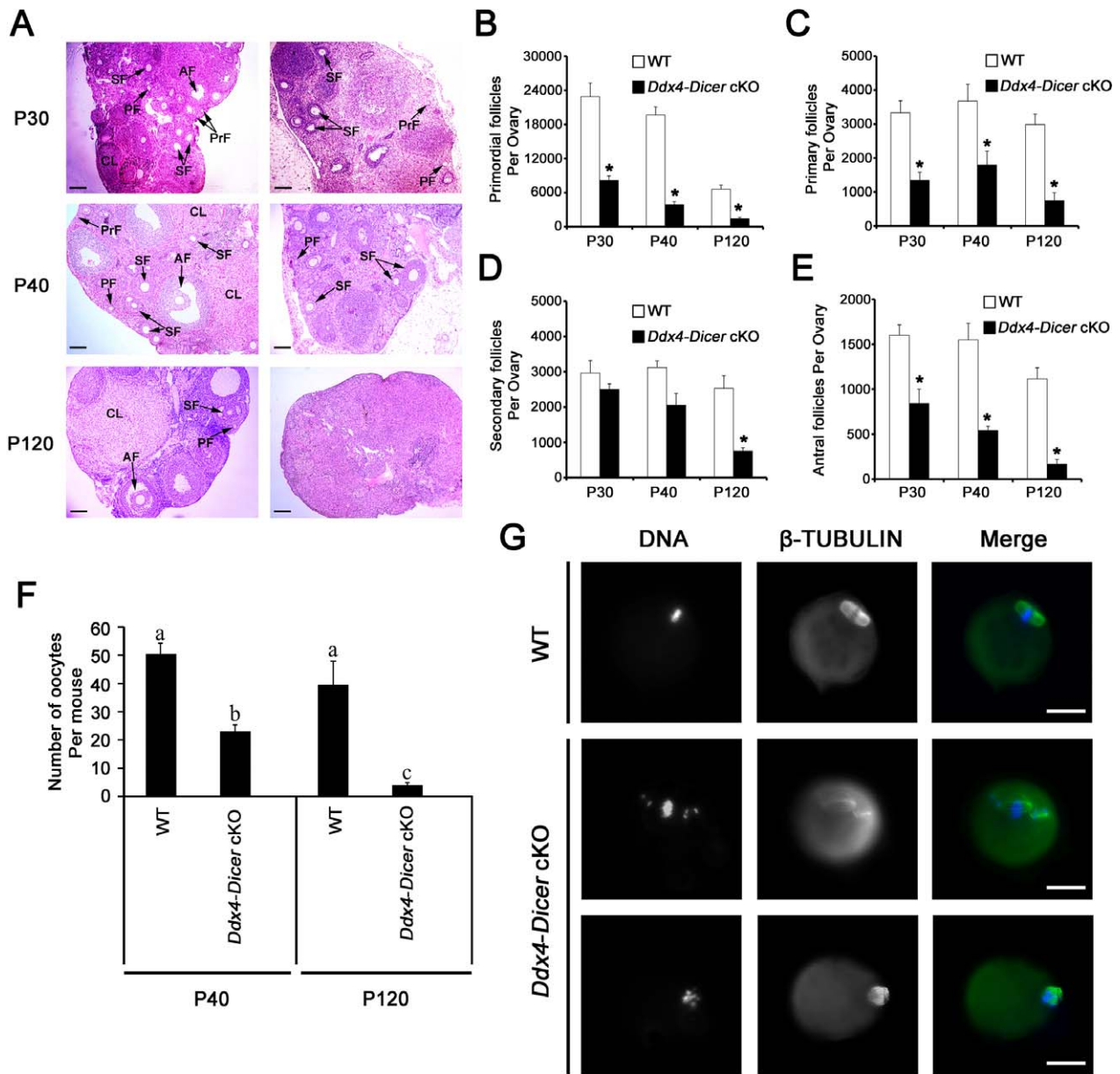


FIG. 3. Lack of *Dicer* in early prophase I oocytes leads to compromised folliculogenesis, premature ovarian failure, and oocyte maturation defects in the adult ovary. **A**) Ovarian histology of WT and *Ddx4-Dicer* cKO females at P30, P40, and P120. H&E-stained cross sections of WT (left panels) ovaries show normal folliculogenesis at all three age groups. In *Ddx4-Dicer* cKO (right panels) ovaries, primordial follicles (PrF), and antral follicles (AF) are rarely seen at P30 and P40, and follicles are largely lacking at P120 (right lower panel). PF, primary follicle; SF, secondary follicle; CL, corpus luteum. Bar = 100 μ m. **B–E**) The numbers of PrF (**B**), PF (**C**), SF (**D**), and AF (**E**) in WT and *Ddx4-Dicer* cKO ovaries at P30, P40, and P120. Bars represent means \pm SEM. * $P < 0.05$ ($n = 3$ for both WT and *Ddx4-Dicer* cKO at P30; $n = 3$ for WT and $n = 4$ for *Ddx4-Dicer* cKO at P40; $n = 3$ for both WT and *Ddx4-Dicer* cKO at P120). **F**) The number of GV-stage oocytes collected from WT and *Ddx4-Dicer* cKO females after superovulation at P40 and P120. Bars represent means \pm SEM ($n = 12$ for WT, $n = 4$ for *Ddx4-Dicer* cKO at P40; $n = 8$ for WT, $n = 3$ for *Ddx4-Dicer* cKO at P120). Data marked with different letters are statistically significant ($P < 0.05$). **G**) Spindle defects in *Ddx4-Dicer* cKO oocytes revealed by immunofluorescent analyses. The spindle was stained with β -tubulin antibody, and DNA was counterstained with DAPI. Representative images are shown. Bar = 50 μ m.

Zp3-Drosha cKO females display no discernible phenotype. Moreover, conditional inactivation of *Drosha* in oocytes of the fetal ovary at E15.5 (*Ddx4-Cre; Drosha^{fllox/fllox}* mice) has no effects on follicular development in postnatal and adult ovaries or on female fertility. Together, these data support the notion that DROSHA and DGCR8 play a similar role, and both are required for embryonic development beyond E6.5, but their expression in oocytes is dispensable for follicular development.

Dicer inactivation in growing oocytes of primary follicles appears to be compatible with folliculogenesis because no abnormalities in ovarian histology and normal superovulation

outcome were observed [20]. However, those *Dicer*-deficient oocytes failed to mature properly, as evidenced by aberrant spindle formation [20], which may have been directly caused by DICER deficiency or have indirectly resulted from disrupted production of DICER-dependent miRNAs and/or endo-siRNAs. In this report, we have demonstrated that oocyte *Dicer* expression in the fetal ovary is essential for postnatal follicular development. The fact that *Ddx4-Dicer* cKO ovaries contain many fewer primordial follicles but display no significantly accelerated primordial recruitment at young (P30 and P40) and adult (P120) ages suggests that the

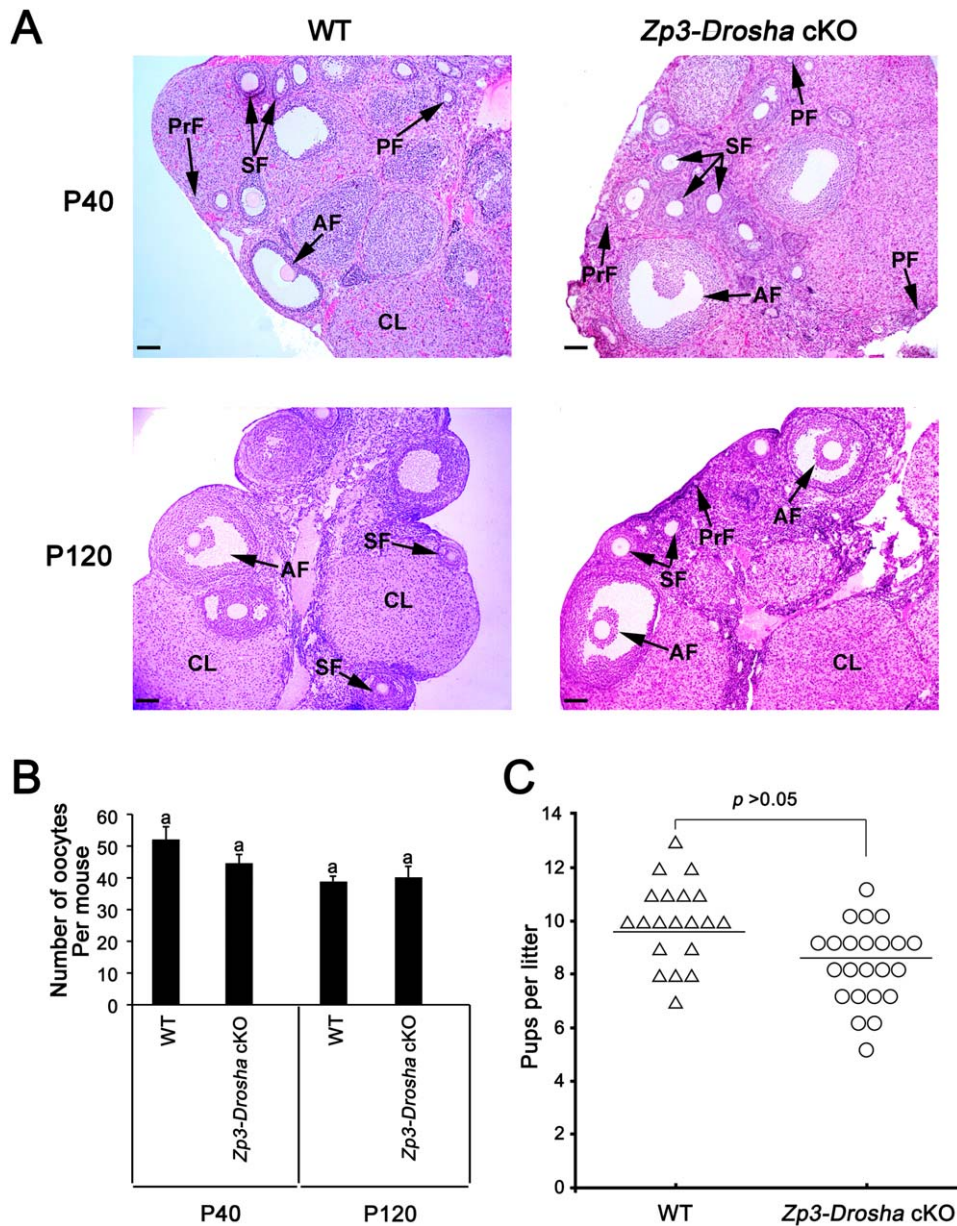


FIG. 4. Oocyte *Drosha* is dispensable for folliculogenesis. **A**) Ovarian histology of WT and *Zp3-Drosha* cKO females at P40 and P120. H&E-stained cross sections of WT (left panels) and *Zp3-Drosha* cKO (right panels) ovaries show normal folliculogenesis at both P40 and P120. PrF, primordial follicles; PF, primary follicle; SF, secondary follicle; AF, antral follicle; CL, corpus luteum. Bar = 100 μ m. **B**) The number of GV-stage oocytes recovered from WT and *Zp3-Drosha* cKO females after superovulation at P40 and P120. Bars represent means \pm SEM ($n = 12$ for WT, $n = 6$ for *Zp3-Drosha* cKO at P40; $n = 8$ for WT, $n = 9$ for *Zp3-Drosha* cKO at P120). Data marked with different letters are statistically significant ($P < 0.05$). **C**) *Zp3-Drosha* cKO females display normal fertility. Adult *Zp3-Drosha* cKO females mated with adult WT males produced a litter size comparable to that of WT control mating pairs. Both individual and mean values are shown ($n = 20$ for WT and $n = 23$ for *Zp3-Drosha* cKO mice).

premature ovarian failure in *Ddx4-Dicer* cKO females is likely caused by reduced primordial follicle formation. Moreover, the significantly reduced number of primary follicles suggests suppressed recruitment of primordial follicles and/or enhanced depletion of primary follicles, and the drastically reduced number of antral follicles may be due to a block in secondary follicle stage given that the number of secondary follicles appeared to decrease at a slower pace from P30 to P40. Together, our data indicate that oocyte *Dicer* expression in the fetal ovary is required for postnatal follicular development both quantitatively and qualitatively. Conditional knockout of *Drosha* in oocytes of either the fetal (*Ddx4-Drosha* cKO) or the postnatal (*Zp3-Drosha* cKO) ovary causes no disruptions in folliculogenesis, which is in sharp contrast to the *Ddx4-Dicer*

cKO females showing compromised folliculogenesis, premature ovarian failure, and female infertility. The phenotypic differences between *Dicer* and *Drosha* cKO females reflect most likely the contribution of their nonoverlapping functions, e.g., endo-siRNA production, or other unknown, DICER-dependent and DROSHA-independent cellular functions. Nevertheless, it remains puzzling that *Drosha*, despite its essential role in miRNA production and the abundant expression of miRNAs in both developing and mature oocytes, is dispensable for oocyte development and fertility.

In summary, our data provide physiological evidence showing that proper *Dicer* expression in early prophase I oocytes of the fetal ovary is essential for normal follicular development in the postnatal ovary. Like DGCR8, DROSHA is

essential for early embryonic development, but its expression in oocytes is dispensable for postnatal follicular development. Further study is needed to fully understand the differential roles of oocyte DICER and DROSHA in sncRNA biogenesis and in other sncRNA-independent functions during folliculogenesis.

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REFERENCES

- Buehr M. The primordial germ cells of mammals: some current perspectives. *Exp Cell Res* 1997; 232:194–207.
- McLaren A. Primordial germ cells in the mouse. *Dev Biol* 2003; 262: 1–15.
- Bendel-Stenzel M, Anderson R, Heasman J, Wylie C. The origin and migration of primordial germ cells in the mouse. *Semin Cell Dev Biol* 1998; 9:393–400.
- Saitou M, Yamaji M. Primordial germ cells in mice. *Cold Spring Harb Perspect Biol* 2012; 4:223–241.
- Jiang JY, Cheung CK, Wang Y, Tsang BK. Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. *Front Biosci* 2003; 8:d222–d237.
- Betgowa A, Smith GW. Mechanisms of maternal mRNA regulation: implications for mammalian early embryonic development. *Front Biosci* 2007; 12:3713–3726.
- Gomes AQ, Nolasco S, Soares H. Non-coding RNAs: multi-tasking molecules in the cell. *Int J Mol Sci* 2013; 14:16010–16039.
- Tesfaye D, Hossain MM, Schellander K. The noncoding genome: implications for ruminant reproductive biology. *Soc Reprod Fertil Suppl* 2010; 67:73–93.
- Schmittgen TD. Regulation of microRNA processing in development, differentiation and cancer. *J Cell Mol Med* 2008; 12:1811–1819.
- Yadav RP, Kotaja N. Small RNAs in spermatogenesis. *Mol Cell Endocrinol* 2014; 382:498–508.
- Babiarz JE, Ruby JG, Wang Y, Bartel DP, Blelloch R. Mouse ES cells express endogenous shRNAs, siRNAs, and other microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev* 2008; 22:2773–2785.
- Wang Y, Medvid R, Melton C, Jaenisch R, Blelloch R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet* 2007; 39:380–385.
- Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet* 2003; 35:215–217.
- Chong MM, Rasmussen JP, Rudensky AY, Littman DR. The RNaseIII enzyme Droscha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med* 2008; 205:2005–2017.
- Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 2009; 10:126–139.
- Yang JS, Lai EC. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Mol Cell* 2011; 43: 892–903.
- Murchison EP, Hannon GJ. miRNAs on the move: miRNA biogenesis and the RNAi machinery. *Curr Opin Cell Biol* 2004; 16:223–229.
- Park CY, Choi YS, McManus MT. Analysis of microRNA knockouts in mice. *Hum Mol Genet* 2010; 19:R169–R175.
- Tang F, Kaneda M, O'Carroll D, Hajkova P, Barton SC, Sun YA, Lee C, Tarakhovskiy A, Lao K, Surani MA. Maternal microRNAs are essential for mouse zygotic development. *Genes Dev* 2007; 21:644–648.
- Murchison EP, Stein P, Xuan Z, Pan H, Zhang MQ, Schultz RM, Hannon GJ. Critical roles for Dicer in the female germline. *Genes Dev* 2007; 21: 682–693.
- Korhonen HM, Meikar O, Yadav RP, Papaioannou MD, Romero Y, Da Ros M, Herrera PL, Toppari J, Nef S, Kotaja N. Dicer is required for haploid male germ cell differentiation in mice. *PLoS One* 2011; 6:e24821.
- Wu Q, Song R, Ortogero N, Zheng H, Evanoff R, Small CL, Griswold MD, Namekawa SH, Royo H, Turner JM, Yan W. The RNase III enzyme DROSHA is essential for microRNA production and spermatogenesis. *J Biol Chem* 2012; 287:25173–25190.
- Papaioannou MD, Lagarrigue M, Vejnar CE, Rolland AD, Kuhne F, Aubry F, Schaad O, Fort A, Descombes P, Neerman-Arbez M, Guillou F, Zdobnov EM, et al. Loss of Dicer in Sertoli cells has a major impact on the testicular proteome of mice. *Mol Cell Proteomics* 2011; 10:M900587MCP900200.
- Kim GJ, Georg I, Scherthan H, Merckenschlager M, Guillou F, Scherer G, Barrionuevo F. Dicer is required for Sertoli cell function and survival. *Int J Dev Biol* 2010; 54:867–875.
- Papaioannou MD, Pitetti JL, Ro S, Park C, Aubry F, Schaad O, Vejnar CE, Kuhne F, Descombes P, Zdobnov EM, McManus MT, Guillou F, et al. Sertoli cell Dicer is essential for spermatogenesis in mice. *Dev Biol* 2009; 326:250–259.
- Romero Y, Meikar O, Papaioannou MD, Conne B, Grey C, Weier M, Pralong F, De Massy B, Kaessmann H, Vassalli JD, Kotaja N, Nef S. Dicer1 depletion in male germ cells leads to infertility due to cumulative meiotic and spermiogenic defects. *PLoS One* 2011; 6:e25241.
- Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD. Dicer1 is required for differentiation of the mouse male germline. *Biol Reprod* 2008; 79:696–703.
- Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, Chen J, Blelloch R. MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr Biol* 2010; 20:271–277.
- Suh N, Blelloch R. Small RNAs in early mammalian development: from gametes to gastrulation. *Development* 2011; 138:1653–1661.
- Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci U S A* 2005; 102:10898–10903.
- Gallardo T, Shirley L, John GB, Castrillon DH. Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre. *Genesis* 2007; 45:413–417.
- de Vries WN, Binns LT, Fancher KS, Dean J, Moore R, Kemler R, Knowles BB. Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis* 2000; 26:110–112.
- Andras Nagy MG, Vintersten K, Behringer R. *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2003:5.
- Tomic D, Brodie SG, Deng C, Hickey RJ, Babus JK, Malkas LH, Flaws JA. Smad 3 may regulate follicular growth in the mouse ovary. *Biol Reprod* 2002; 66:917–923.
- Lira SA, Kinloch RA, Mortillo S, Wassarman PM. An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. *Proc Natl Acad Sci U S A* 1990; 87: 7215–7219.
- Lan ZJ, Xu X, Cooney AJ. Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. *Biol Reprod* 2004; 71:1469–1474.
- Tijsterman M, Plasterk RH. Dicers at RISC: the mechanism of RNAi. *Cell* 2004; 117:1–3.
- Macias S, Plass M, Stajuda A, Michlewski G, Eyraes E, Caceres JF. DGCR8 HITS-CLIP reveals novel functions for the microprocessor. *Nat Struct Mol Biol* 2012; 19:760–766.