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ORIGINAL RESEARCH

Germ stem cells are active in postnatal mouse ovary under physiological conditions

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STUDY HYPOTHESIS: Are active ovarian germ stem cells present in postnatal mouse ovaries under physiological conditions?

STUDY FINDING: Active ovarian germ stem cells exist and function in adult mouse ovaries under physiological conditions.

WHAT IS KNOWN ALREADY: *In vitro* studies suggested the existence of germ stem cells in postnatal ovaries of mouse, pig and human. However, *in vivo* studies provided evidence against the existence of active germ stem cells in postnatal mouse ovaries. Thus, it remains controversial whether such germ stem cells really exist and function *in vivo* in postnatal mammalian ovaries.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Octamer-binding transcription factor 4 (*Oct4*)-MerCreMer transgenic mice were crossed with *R26R*-enhanced yellow fluorescent protein (*EYFP*) mice to establish a tamoxifen-inducible tracing system so that *Oct4*-expressing potential ovarian germ stem cells in young adult mice (5–6 weeks old) can be labeled with *EYFP*. The germ cell activities of DNA replication, mitotic division, entry into meiosis and progression to primordial follicle stage were investigated by means of immunofluorescent staining of ovarian tissues collected at different time points post-tamoxifen injection (1 day, 3 days, 2 months and 4 months). Meiosis entry and primordial follicle formation were also measured by *EYFP*-labeled single-cell RT–PCR. Germ cell proliferation and mitotic division were examined through 5-bromodeoxyuridine triphosphate incorporation assay. At each time point, ovaries from two to three animals were used for each set of experiment.

MAIN RESULTS AND THE ROLE OF CHANCE: By labeling the *Oct4*-expressing small germ cells and tracing their fates for up to 4 months, we observed persistent meiosis entry and primordial follicle replenishment. Furthermore, we captured the transient processes of mitotic DNA replication as well as mitotic division of the marked germ cells at various time periods after tracing. These lines of evidence unambiguously support the presence of active germ stem cells in postnatal ovaries and their function in replenishing primordial follicle pool under physiological conditions. Moreover, we pointed out that $Oct4^{+}$ deleted in azoospermia-like $(Dazl)^{-}$ but not $Oct4^{+}Dazl^{+}$ or $Oct4^{+}$ DEAD (Asp–Glu–Ala–Asp) Box Polypeptide 4 $(Ddx4)^{+}$ cells contain a population of germ stem cells in mouse ovary.

LIMITATIONS, REASONS FOR CAUTION: This study was conducted in mice. Whether or not the results are applicable to human remain unclear. The future work should aim at identifying the specific ovarian germ stem cell marker and evaluating the significance of these stem cells to normal ovarian function.

WIDER IMPLICATIONS OF THE FINDINGS: Clarifying the existence of active germ stem cells and their functional significance in postnatal mammalian ovaries could provide new insights in understanding the mechanism of ovarian aging and failure.

LARGE SCALE DATA: Not applicable.

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Key words: genetic tracing / primordial follicle replenishment / ovarian germ stem cells / postnatal mouse ovary / meiosis / neo-oogenesis

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Introduction

Some invertebrate (e.g. Drosophila) and lower vertebrate females (e.g. fish) continuously produce gametes from germ stem cells in ovaries after birth (Draper *et al*., 2007; Xie *et al*., 2008; Nakamura *et al*., 2010). In mammals, the prevailing dogma in the field declares that neo-oogenesis ceases at birth as oocytes enter the prophase of the first meiotic division and arrest at the dictyate. Under this rubric, newborn females possess their life time complement of germ cells stored as primordial follicles that are not replenished after birth. Recently, this model has been challenged by several important findings, which reported the observation of meiosis entry in postnatal mouse ovary (Johnson *et al*., 2004), or isolating rare cells in adult mouse and human ovarian tissue that can proliferate in dishes and form developmentally competent oocytes following transplantation (Zou *et al*., 2009; White *et al*., 2012). Although these pioneer works and other follow-up studies (Pacchiarotti *et al*., 2010; Zou *et al*., 2011; Bai *et al*., 2013; Bui *et al*., 2014; Zhou *et al*., 2014) suggest the presence of germ stem cells in postnatal ovaries of several mammalian species, they do not address whether such stem cells actually exist and function *in vivo* under physiological conditions. Instead, they reignited debate regarding if these stem cells were an artifact of purification or culture methods (Telfer and Albertini, 2012; Vogel, 2012; Evron and Blumenfeld, 2013; Hernandez *et al*., 2015). For instance, by reconstructing female cell lineage trees, Shapiro *et al*. offered indirect evidence supporting the postnatal oocyte renewal from germ-line stem cells under physiological conditions (Reizel *et al*., 2012; Woods *et al*., 2012). On the other hand, several recent studies presented evidence indicating no germ stem cell activity in postnatal mouse ovaries under physiological and injury conditions (Lei and Spradling, 2013a; Zhang *et al*., 2014, 2015). Thus, whether there are active germ stem cells and primordial follicle replenishment in postnatal ovaries in mammals remains controversial.

In vivo genetic cell fate tracing using Cre-loxP system is a definitive method for investigating the contribution of stem cells to tissue development and homeostasis (Kretzschmar and Watt, 2012). It provides information on how cells behave in the context of intact tissue, as opposed to what can be learned about the cells following their isolation, *in vitro* culture and transplantation. The key to successful genetic cell fate tracing is the selection of the gene driving Cre recombinase expression. Ideally, stem-cell-specific gene is used to drive the tracing system. However, other genes that are not restrictively expressed in stem cells of the investigated tissue can also be utilized in case of lacking stem cell marker (Morris *et al*., 2004; Kretzschmar and Watt, 2012). To investigate whether the putative germ stem cells function *in vivo* in adult mouse ovaries, one recent work employed CAG (chicken *β-actin*) promoter to drive the Cre-loxP system in a tamoxifen-inducible manner. They observed a stable primordial follicle pool, but did not detect the presence of DEAD (Asp–Glu–Ala–Asp) box polypeptide 4 (*Ddx4*)-expressing germ cell clusters, which they considered as an indicator of the existence of active germ stem cells. Therefore, they concluded that adult female mice lacked germ stem cells but sustained oogenesis using stable primordial follicles (Lei and Spradling, 2013a). Germ-cell-specific gene *Ddx4* (also known as *Vasa*) is considered to be expressed and localized in plasma membrane of putative germ stem cells of postnatal ovaries. Its extracellular epitope was utilized to isolate a germ stem cell population in previous studies (Zou *et al*., 2009; White *et al*., 2012; Imudia *et al*., 2013). However, *Ddx4* expression is not detected in undifferentiated primitive germ cells of fetal ovaries (Toyooka *et al*., 2000; Anderson *et al*., 2007). Utilizing *Ddx4*-driving Cre-loxp system to label the *Ddx4*-expressing cells also failed to detect mitotic cell division of such cells or *de novo* folliculogenesis in mice (Zhang *et al*., 2012). Moreover, recent two studies challenged the idea that *Ddx4* extracellular epitope can be used to isolate putative ovarian germ stem cells (Hernandez *et al*., 2015; Zhang *et al*., 2015). Thus, whether putative ovarian germ stem cells express *Ddx4* or if the *Ddx4*-driven Cre-loxp system can mark ovarian germ stem cells are questioned (Bhartiya *et al*., 2013). Freshly purified putative germ stem cells from postnatal mouse ovary and undifferentiated primitive germ cells in fetal ovary express octamer-binding transcription factor 4 (*Oct4*) (Bowles and Koopman, 2007; Imudia *et al*., 2013). Therefore, we used *Oct4* promoter to drive a tamoxifen-inducible Cre-loxP tracing system to investigate whether there are active germ stem cells in postnatal mouse ovaries under physiological conditions.

Materials and Methods

Mice

Normal CD1 female mice (3–4 weeks old) were used for these investigations. Mouse lines *Oct4*-MerCreMer, *R26R*-enhanced yellow fluorescent protein (*EYFP*) and OG2 were obtained from the Jackson Laboratory (Bar Harbor, ME, USA; stock numbers 016829, 006148, and 004654, respectively, C57BL/6 background). Mice were maintained in specific-pathogen-free condition. *Oct4*-MerCreMer mice were crossed with *R26R-EYFP* mice. *Oct4*-MerCreMer$^{+/Tm}$; *R26R-EYFP*$^{Tm/Tm}$ female and male mice (5–6 weeks old) were used for tamoxifen injection.

Ethics

Animal care and experimental procedures were approved by the Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences. The experiments were carried out in accordance with the approved guidelines.

Tamoxifen preparation and injection

Mice were weighed and the amount of tamoxifen (Sigma-Aldrich, St. Louis, MO, USA; T5648) required for administration was calculated according to the working concentration (e.g. 0.25 mg/g body weight). Tamoxifen, which was sufficient for a single injection, was dissolved in 300 μl 100% ethanol. Corn oil (300 μl) (Sigma-Aldrich, St. Louis, MO, USA) was then added into the tamoxifen ethanol solution to make a working solution. The mixture of tamoxifen-containing ethanol and corn oil was vortexed and pipetted up and down to eliminate precipitation. Residual ethanol was removed by vacuum centrifuge. The whole of the corn-oil solution tamoxifen was then administered to a mouse by intraperitoneal injection (i.p. injection).

Frozen tissue section preparation and immunofluorescence staining

Ovarian and testis tissues were fixed in formalin (10%, v/v, buffered neutral) at room temperature for 14 h, followed by incubation in 15 (w/v) and 30% (w/v) sucrose overnight and embedded in O.C.T. (Tissue Freezing Medium, Leica, Wetzlar, Germany; 020108926). The specimens were stored at −80°C before cutting 8-μm sections. For immunofluorescence staining, antigen retrieval was performed by placing slides in a pressure cooker in citrate antigen retrieval buffer (pH 6.0) (Fuzhou Maixin Biotech, Fuzhou, China; MVS-0100) for 2 min. Slides were then washed in phosphate buffered saline (PBS) and permeabilized in 0.1% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Sections were blocked

with 5% (w/v) bovine serum albumin (BSA) (Sangon, Shanghai, China; A0332) followed by sequential incubations with primary and secondary antibodies (Table I). The specificity of all primary antibodies was examined by omitting primary antibodies in immunostaining. DNA was counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). Mounted slides were imaged using a laser scanning confocal microscope (Olympus, Tokyo, Japan). Images were analyzed using the FV10-ASW 2.1 viewer software. For each experiment, testis or ovaries from two to three animals were used for investigation.

5-Bromo-2′-deoxyuridine triphosphate incorporation assay

BrdU (5-Bromo-2′-deoxyuridine; Sigma-Aldrich, St. Louis, MO, USA) was re-suspended in PBS before administration. BrdU solution was injected i.p. into 3- to 4-week-old CD1 females (50 mg/kg body weight) and tamoxifen-treated double-mutant mice. Ovarian tissues were collected 2 (for CD1 mice) or 12 h (for tamoxinfen treated mice) after BrdU injection and processed for frozen sections as described above. To probe with BrdU primary antibody, sections were sequentially treated with 2N HCl for 30 min and 0.1 M sodium borate for 10 min prior to permeabilization and immunofluorescence staining.

Isolation of *EYFP*-expressing ovarian germ cells

Tamoxifen was injected into 5- to 6-week-old double-targeted mutation mice (*Oct4*-MerCreMer$^{+/Tm}$; *R26R-EYFP*$^{Tm/Tm}$). Ovarian tissues were collected at 1 day, 3 days, 2 weeks and 1 month after tamoxifen injection. Ovarian single-cell suspension was prepared by two-step enzymatic digestion described previously (Zou *et al.*, 2009). In brief, ovaries were placed in PBS solution without calcium or magnesium but containing 1 mg/ml type IV collagenase (Life Technologies, Carlsbad, CA, USA) and incubated at 37°C for 15 min. After washing two times in PBS, ovarian tissue was placed in PBS containing 0.05% (w/v) trypsin (Life Technologies, Carlsbad, CA, USA.) and incubated at 37°C for 5–10 min. Trypsin was neutralized by adding 10% (v/v) fetal bovine serum. The clumps of residual tissue were removed by passing the suspension through a 70-μm nylon cell strainer. The cell suspension was centrifuged at 300*g* for 5 min. The pellet was re-suspended in 0.3% (w/v) PBS-BSA. *EYFP*-expressing germ cells were then manually picked by using a mouth pipette under the fluorescent microscope (Nikon Eclipse Ti-S, Tokyo, Japan).

Single-cell cDNAs preparation and amplification

Single-cell cDNAs preparation and amplification were performed according to the published protocol (Tang *et al.*, 2010). Briefly, cDNAs were prepared from single cell and amplified by 20 cycles of PCR. cDNA samples were then stored at −80°C.

Reverse transcription and polymerase chain reaction

PCR primers to detect gene expression are described in Table II. Upper and lower primers of all genes except for *EYFP* were designed to span across introns. The PCR condition consists of 1 cycle at 94°C for 5 min; 35 cycles at

Table I Information of primary and secondary antibodies.

Antibody	Company	Catalog number	Dilution
Primary antibodies			
Oct4	Abcam	AB19857	1:300
Oct4	Santa Cruz	SC8628	1:200
Sycp3	Abcam	AB97672	1:300
Stra8	Abcam	AB49602	1:300
Ddx4	Abcam	AB13840	1:300
GFP	Abcam	AB13970	1:2000
GFP	Cell Signaling Technology	2956	1:200
BrdU	Novus Biologicals	NB500-169	1:300
Dazl	Novus Biologicals	NB100-2437	1:300
Stella	Abcam	AB19878	1:300
Foxl2	Abcam	AB5096	1:200
Secondary antibodies			
Alexa Fluor® 488 Donkey anti Rabbit IgG	Invitrogen	A-21206	1:500
Alexa Fluor® 555 Donkey anti Rabbit IgG	Invitrogen	A-31572	1:500
Alexa Fluor® 488 Goat anti Rabbit IgG	Invitrogen	A-11008	1:500
Alexa Fluor® 647 Goat anti Rabbit IgG	Invitrogen	A-21244	1:500
Alexa Fluor® 555 Donkey anti Goat IgG	Invitrogen	A-21432	1:500
Alexa Fluor® 488 Goat anti Mouse IgG	Invitrogen	A-11029	1:500
Alexa Fluor® 555 Donkey anti Mouse IgG	Invitrogen	A-31570	1:500
Alexa Fluor® 488 Goat anti Chicken IgG	Invitrogen	A-11039	1:500
Alexa Fluor® 488 Donkey anti Rat IgG	Invitrogen	A-21208	1:500
Alexa Fluor® 647 Goat anti Rat IgG	Invitrogen	A-21247	1:500

Table II Details for PCR primers used to analyze gene expression in cDNA samples prepared from mouse cells and tissues.

Gene	Accession number	Primer sequences (5′ to 3′; F, forward; R, reverse)	Size (bp)
Oct4	NM_013633	F: GGAGGAAGCCGACAACAA	322
		R: GGGCAGAGGAAAGGATACAG	
Stella	NM_139218	F: ATGAAGAGGACGCTTTGGA	382
		R: CCGATTTTCGCATTCTCAG	
Ddx4	NM_010029	F: CATTCCCATTGTATTAGCAGG	481
		R: AAGGGTTTGGCGTTGTTC	
Dazl	NM_010021	F: CTCCTCCACCACAGTTCCA	365
		R: CACTGTCTGTATGCTTCGGTC	
Sycp3	NM_011517	F: AGCCACCTTTGGTTGATC	497
		R: CTGCTCGTGTATCTGTTTGA	
Stra8	NM_009292	F: ACAACCTAAGGAAGGCAGTTTAC	173
		R: GACCTCCTCTAAGCTGTTGGG	
EYFP	KP171163	F: CACAAGTTCAGCGTGTCCG	421
		R: AGTTCACCTTGATGCCGTTC	
Gapdh	NM_008084	F: TTGAGGTCAATGAAGGGGTC	132
		R: TCGTCCCGTAGACAAAATGG	
Sohlh1	NM_001001714.1	F: GATGTCTGTGTACTTCCTCC	320
		R: CTGGCTCACTGAATGACAAC	

94°C for 30 s, 57°C for 40 s, and 72°C for 40 s; and an extension cycle at 72°C for 10 min. When amplifying synaptonemal complex protein 3 (*Sycp3*), stimulated by retinoic acid gene 8 (*Stra8*), spermatogenesis- and oogenesis-specific basic helix–loop–helix 1 (*Sohlh1*) and *EYFP*, the annealing temperature was set at 54°C.

The following controls were set up in performing PCR: cDNA templates from mouse testis were used as positive controls in detecting *Sycp3*, *Stra8* and *Sohlh1* mRNA expression; cDNAs from single germinal vesicle stage oocyte were used as positive controls for detection of *Oct4*, developmental pluripotency-associated protein 3 (*Stella*), deleted in azoospermia-like (*Dazl*), and *Ddx4* expression; genomic DNAs prepared from *R26R-EYFP*$^{Tm/Tm}$ mouse tail tip were used as positive control for detection of *EYFP*. cDNA samples from somatic cells were used as negative control in detection of germ-cell-specific gene expression.

Results

Oct4 is expressed in primitive germ cells of postnatal mouse ovaries and can be used for marking putative germ stem cells

Initiation of female germ cell differentiation and entrance into meiosis occur prior to follicle formation. Previous studies reported the detection of small germ cells not intimately associated with a layer of squamous epithelial cells in the surface epithelium, resembling germ cells in fetal ovaries (Johnson *et al.*, 2004; Niikura *et al.*, 2009). Importantly, some of these small germ cells were capable of DNA replication or expressing *Sycp3* indicative of the meiosis entry (Johnson *et al.*, 2004; Zou *et al.*, 2009). We validated these observations by immunostaining ovaries of juvenile mice (3–4 weeks old, 2–3 animals for each group of immunostaining examination) with germ cell differentiation markers *Dazl*, *Ddx4*, *Stra8* and *Sycp3*. The specificity of the commercial antibodies to *Stra8* and *Sycp3* was verified in testicular samples (Supplementary Fig. S1). It should be mentioned that the protein distribution pattern of *Sycp3* in ovary is distinct from that in testis. As previously reported (Yang *et al.*, 2006; Morelli *et al.*, 2008; Tsutsumi *et al.*, 2011), *Sycp3* aligns on chromosomes in testis (Supplementary Fig. S1a). However, it is distributed as punctuates in ovary (Supplementary Fig. S1e) (Liu *et al.*, 2007; Zou *et al.*, 2009; Lei and Spradling, 2013b). Consistently, a population of small germ cells not morphologically in any type of follicular structure were detected at the ovarian surface (refer to as small germ cells thereafter, 98% of them have diameter <15 μm, $n = 1476$) (Fig. 1a). Co-staining these cells with antibody to *Oct4*, a pluripotency and germ-line-specific marker expressed in primitive germ cells and freshly isolated putative ovarian germ stem cells (Scholer *et al.*, 1989; Bowles and Koopman, 2007; Imudia *et al.*, 2013), documented their heterogeneity in developmental stage (Fig. 1a and b). For instance, co-staining of *Oct4* with *Stra8* revealed three populations. Among them, *Oct4*$^+$*Stra8*$^-$ (5.61%, $n = 107$) germ cells were more primitive than *Oct4*$^+$*Stra8*$^+$ (63.55%, $n = 107$) followed by *Oct4*$^-$stra8$^+$ (30.84%, $n = 107$) germ cells. Importantly, a minority of the cells were small (5–10 μm in diameter), expressed *Oct4*, but did not initiate the early germ cell differentiation marker *Dazl* or *Ddx4* (*Oct4*$^+$*Dazl*$^-$ or *Oct4*$^+$*Ddx4*$^-$) (Fig. 1a), and thus represented the most primitive germ cells in postnatal ovaries. To confirm the germ cell identity of these *Oct4*$^+$*Dazl*$^-$ or *Oct4*$^+$*Ddx4*$^-$ cells, we examined whether these *Oct4*-expressing cells express another primitive germ cell marker *Stella* (Saitou *et al.*, 2002; Payer *et al.*, 2003). To do this, we used the OG2-enhanced green fluorescent protein (*EGFP*) transgenic mice (3–4 weeks old) in which *EGFP* expression is driven by germ-line-specific *Oct4* promoter (Yeom *et al.*, 1996). Triple staining with *EGFP*, *Stella* and *Dazl* revealed that all *EGFP*/*Oct4*-expressing cells are germ cell lineage as evidenced by the expression of germ cell marker *Dazl* and/or *Stella* (Fig. 1c, 288 *EGFP*$^+$ small germ cells were examined in total). Moreover, co-staining of OG2-*EGFP* mouse

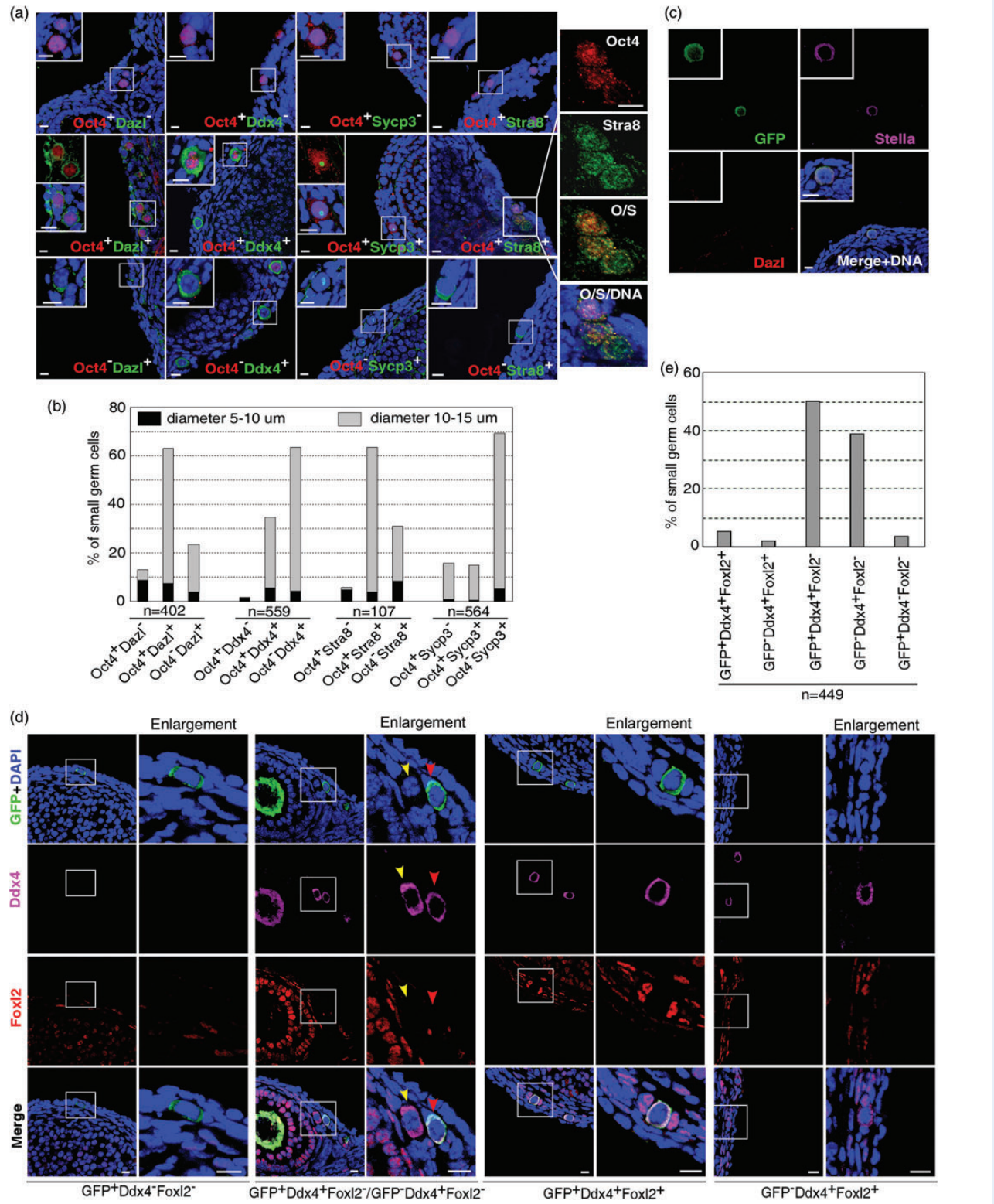

Figure 1 Small germ cells at differential developmental stages in juvenile (3–4 weeks old) mouse ovaries. (**a**) Co-staining *Oct4* with *Dazl*, *Ddx4*, *Sycp3* or *Stra8* documented different types of small germ cells. Insets show the higher magnification of germ cells indicated by squares. (**b**) Proportions of each type of small germ cells. (**c**) In OG2 transgenic mouse ovaries, $EGFP^{+}Dazl^{-}$ ($n = 288$) cells express germ cell marker *Stella*, indicating that $Oct4^{+}Dazl^{-}$ cells are germ cells. (**d**) Co-staining of OG2-*EGFP* mouse ovaries with *EGFP*, *Ddx4* and *Foxl2* documented five types of small germ cells. Enlargement columns show the higher magnification of germ cells in squares. Red arrowhead indicates the $EGFP^{+}Ddx4^{+}$ germ cell not surrounded by *Foxl2*-expressing granulosa cells. Yellow arrowhead, the $EGFP^{-}Ddx4^{+}$ small germ cell not surrounded by *Foxl2*-expressing granulosa cells. (**e**) Proportions of each type of small germ cells in OG2 transgenic mouse ovaries according to the expression of *EGFP*, *Ddx4* and *Foxl2*. Ovaries from two to three animals were used for each group of immunostaining. Scale bar, 10 μm.

ovaries with *EGFP* (*Oct4*), *Ddx4*, and forkhead box L2 (*Foxl2*), a marker of the granulosa cells (Schmidt *et al*., 2004; Matson *et al*., 2011; Zhang *et al*., 2014), showed that the majority (92.4%, $n = 449$) of the small germ cells (not morphologically in any type of follicular structure) was not surrounded by *Foxl2*-expressing granulosa cells (Fig. 1d and e). These results validated that >90% of small germ cells were indeed at pre-follicular stage. In particular, all *EGFP*(*Oct4*)$^+$*Ddx4*$^-$ small germ cells did not form a follicle (Fig. 1d and e). Taken together, these observations suggested the presence of primitive germ cells and proposed the use of *Oct4* promoter to establish a Cre-loxP genetic tracing system for labeling putative germ stem cells and investigating their physiological activities in postnatal mouse ovary.

Cre-loxP genetic tracing of *Oct4*-expressing cells in postnatal mouse ovaries reveals persistent meiosis entry and formation of new primordial follicles

Oct4-MerCreMer mice were crossed with *R26R-EYFP* mice to establish a tamoxifen-inducible tracing system in which cells expressing *Oct4* at the time of tamoxifen administration are permanently marked with *EYFP* (Supplementary Fig. S2). Because *Oct4* is expressed in mouse spermatogonial stem cells (SSCs) (Ohbo *et al*., 2003), we first tested whether this cell tracing system functioned in males. No germ cells in testis expressed *EYFP* without tamoxifen induction, indicating the absence of constitutive expression or of system leak (Supplementary Fig. S3a). At 3 days post-injection (dpi) of tamoxifen (0.25 mg/g body weight), some *EYFP*$^+$ germ cells were specifically detected near the basal membrane of seminiferous tubule (Supplementary Fig. S3b and c). Given that the completion of spermatogenesis takes about 1 month in mice, we examined the fates of *EYFP*$^+$ cells at 1 and 4 months post-injection (mpi). *EYFP*$^+$ germ cells at differential spermatogenic stages were observed at both time points (Supplementary Fig. S3d–g), indicating continuous spermatogenesis from the *EYFP*-labeled SSCs and the soundness of the tracing system.

In postnatal ovaries, *Oct4* is expressed not only in a fraction of non-follicular germ cells (Fig. 1), but also in oocytes as they progress through folliculogenesis. Thus, *Oct4*-expressing germ cells prior to follicle formation and within follicles can be marked with *EYFP*. Nevertheless, the initiation and entry into meiosis, which are defined by induction of *Stra8* and *Sycp3* expression, occurs exclusively in non-follicular germ cells. We therefore focused on the *EYFP*$^+$ small germ cells. It should be noted that meiosis markers *Stra8* and *Sycp3* are temporarily expressed during prophase I and progressively degraded upon oocytes entering into dictyate arrest (Johnson *et al*., 2004; Prieto *et al*., 2004; Krentz *et al*., 2011). As a result, *Stra8* and *Sycp3* cannot be carried over in germ cells for long time and only germ cells that newly enter into meiosis are responsive for *Stra8* or *Sycp3* immunostaining. We first examined the influence of tamoxifen dosages on labeling efficiency in order to define a suitable dose that could specifically label *Oct4*$^+$*Sycp3*$^-$ small germ cells. Double-mutant female mice (5–6 weeks old) were injected with tamoxifen at the following dosages: 0.25, 0.125, 0.06, 0.03, and 0.01 mg/g body weight. Consistently, no cells in ovaries expressed *EYFP* without tamoxifen induction. All dosages generated similar proportion of *EYFP*$^+$*Sycp3*$^+$ or *EYFP*$^+$*Stra8*$^+$ cells within the entire *EYFP*$^+$ small germ cells at 1 dpi (Supplementary Fig. S4a and b), although the total number of *EYFP*$^+$ small germ cells decreased with the reduction of injection dose. Specifically, at the dose of 0.25 mg/g body weight, 100% ($n = 157$) of *Oct4*-expressing small germ cells were labeled with *EYFP*. We therefore utilized this dosage in all subsequent cell tracing studies. Furthermore, the germ cell identity of the *EYFP*-labeled cells was once again verified by triple staining of ovarian sections collected at 3 dpi of tamoxifen with antibodies against *EYFP*, *Dazl* and *Stella*. All *EYFP*$^+$ cells ($n = 768$) were positive for *Dazl* or/and *Stella*, indicative of germ cell identity (Supplementary Fig. S4c).

At 1 dpi, the majority of *EYFP*-labeled small germ cells still retained *Oct4* staining (77.53%, $n = 832$), ~70% of *EYFP*$^+$ small germ cells ($n = 381$) expressed *Sycp3* (*EYFP*$^+$*Sycp3*$^+$) (Fig. 2a and g). This population displayed a slight increase to 80% at 3 dpi ($n = 212$), suggesting that additional small germ cells moved from *EYFP*$^+$*Sycp3*$^-$ to *EYFP*$^+$*Sycp3*$^+$ status (Fig. 2b and g). *Sycp3* protein was also occasionally detected in some *EYFP*$^+$ germ cells of primordial follicles, indicating that these primordial follicles were newly formed (Fig. 2c). Due to the temporary expression property of *Sycp3* and *Stra8*, after a long time period of tracing, these *EYFP*$^+$*Sycp3*$^+$ germ cells would shift to *EYFP*$^+$*Sycp3*$^-$ due to the degradation of *Sycp3*. Genetic tracing was then extended for 2 months, which is long enough for *Sycp3* to undergo degradation. Notably, we persistently observed *EYFP*$^+$*Sycp3*$^+$ small germ cells, which accounted for 58% of total *EYFP*$^+$ small germ cells ($n = 330$) (Fig. 2a and g). Moreover, a portion of *EYFP*$^+$ germ cells within primordial follicle retained an obvious *Sycp3* signal, indicating that these *EYFP*-marked primordial follicles are newly formed (Fig. 2c). These data suggested the new meiosis entry and primordial follicle replenishment in postnatal ovaries. To confirm the meiosis entry and follicle replenishment, we prolonged genetic tracing to 4 months and obtained similar results (Fig. 2a–c and g). We repeated the tracing experiments by examining the expression of another temporary meiosis marker *Stra8*. Consistently, the *EYFP*$^+$*Stra8*$^+$ small germ cells and primordial follicles containing *EYFP*$^+$*Stra8*$^+$ germ cells were persistent after 2 and 4 months of genetic tracing (Fig. 2d–g). Of note, the percentages of *EYFP*$^+$*Sycp3*$^+$ or *EYFP*$^+$*Stra8*$^+$ small germ cells remained relatively stable at all examined time points (Fig. 2g). This suggested that entry into meiosis might take place in a steady state manner in young adult females. Similarly, the proportions of newly formed *EYFP*$^+$ primordial follicles among the total *EYFP*-labeled primordial follicles (ratio of primordial follicles containing *EYFP*$^+$*Stra8*$^+$ or *EYFP*$^+$*Sycp3*$^+$ germ cells to those containing *EYFP*-labeled germ cells) were constant at 2 and 4 mpi (~45%, $n > 100$). At all time points, the *EYFP*$^+$ oocytes in various follicle stages were observed (Supplementary Fig. S4d–h). No granulosa cells expressed *EYFP* (Supplementary Fig. S4d–h), confirming that *Oct4*-expressing stem cells contribute to germ cell lineage in folliculogenesis.

To further validate the persistent existence of *EYFP*$^+$*Sycp3*$^+$ and *EYFP*$^+$*Stra8*$^+$ germ cells and continuous primordial follicle replenishment in ovaries in our tracing experiment, we isolated *EYFP*-expressing single germ cells with diameters of 5–15 μm at 1 day, 3 days, 2 weeks and 1 month post-tamoxifen injection (Fig. 3a). cDNA was prepared and amplified from single cells according to the published protocol (Tang *et al*., 2010). The mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), *EYFP*, *Oct4*, *Stella*, *Dazl*, *Ddx4*, *Stra8*, *Sycp3* and *Sohlh1* were examined by PCR. As shown (Fig. 3b), isolated *EYFP*-positive germ cells expressed *Dazl* mRNAs, indicating that they initiated differentiation process. Note that some *EYFP*$^+$ germ cells (S5, S6, S7 and S8 in Fig. 3b) showed no or very little *Oct4* mRNAs, indicating that they lost *Oct4* expression along with differentiation (Pesce *et al*., 1998; Kehler *et al*., 2004; Anderson *et al*., 2007; Kretzschmar and Watt, 2012). Interestingly, these germ cells displayed four types of mRNA expression

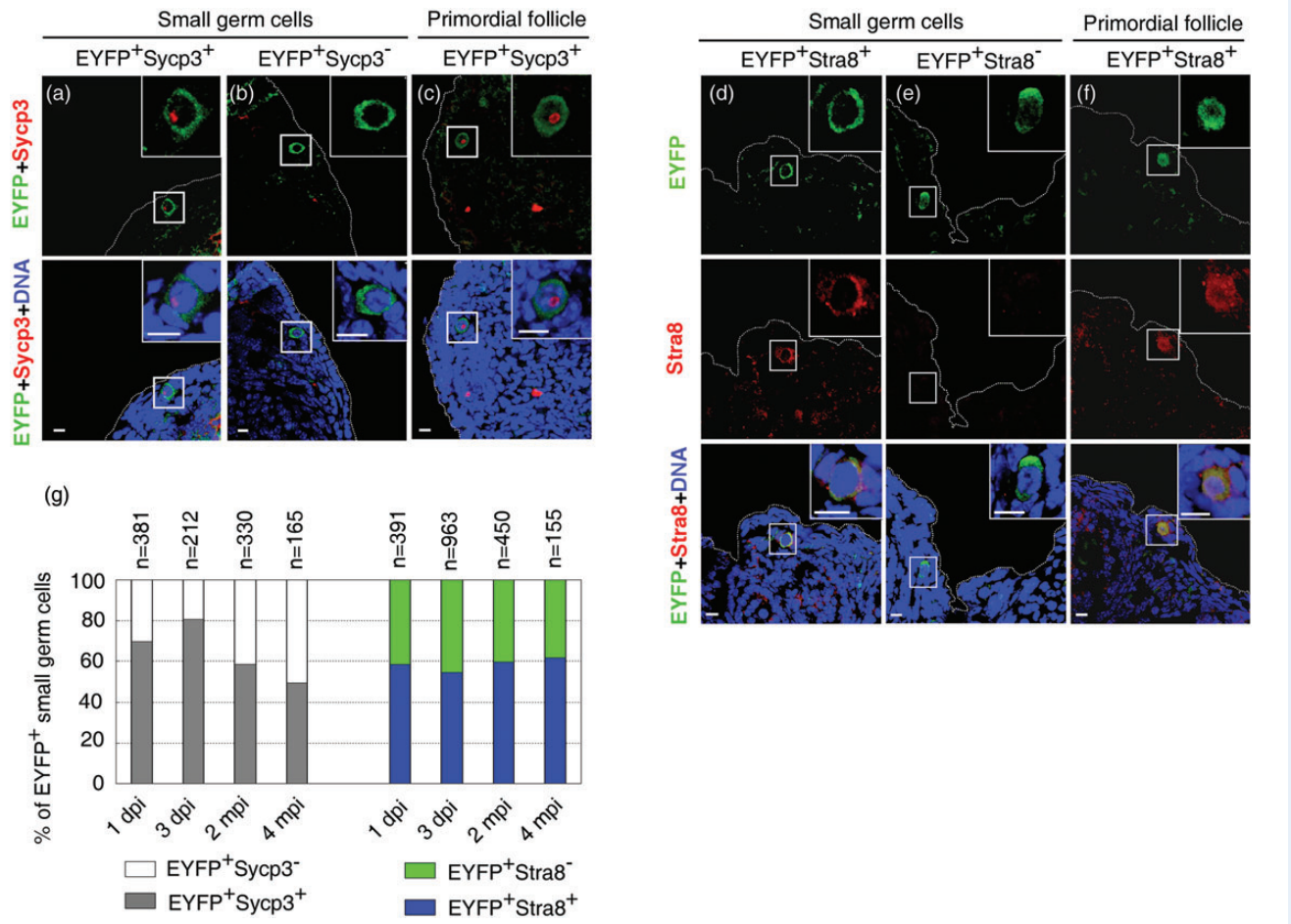

Figure 2 Genetic tracing combined with immunostaining documents continuous meiosis entry and primordial follicle replenishment in postnatal mouse ovaries. Meiosis entry was persistently detected in postnatal mouse ovaries (5–6 weeks old) at 1 and 3 dpi, and 2 and 4 mpi of tamoxifen induction. Displays are representative data collected at 4 mpi. (**a**, **b**, **d** and **e**) Representative micrographs of *EYFP*$^+$ small germ cells expressing *Sycp3* or *Stra8*. (**c** and **f**) Newly formed primordial follicles retaining *Sycp3* or *Stra8*. (**g**) Percentages of *EYFP*$^+$*Sycp3*$^+$ or *EYFP*$^+$*Stra8*$^+$ small germ cells at each time point following tamoxifen injection. Insets show the higher magnification of germ cells indicated by squares. At each time point, ovaries from two to three animals were used for investigation. Scale bar, 10 μm.

pattern for *Stra8* and *Sycp3*, including *Stra8*$^+$*Sycp3*$^-$ (S3 in Fig. 3b), *Stra8*$^-$*Sycp3*$^+$ (S2, S4, S6 and S10 in Fig. 3b), *Stra8*$^+$*Sycp3*$^+$ (S1, S5 and S9 in Fig. 3b), and *Stra8*$^-$*Scyp3*$^-$ (S7 and S8 in Fig. 3b). Furthermore, some *EYFP*$^+$ germ cells not only expressed a marker of primordial follicle *Sohlh1* (Pangas *et al*., 2006; Zhang *et al*., 2014), but also retained *Sycp3* and *Stra8* expression (S5 and S6 in Fig. 3b). These expression patterns were persistently observed in individual *EYFP*-expressing germ cells at all time points examined. Thus, these data altogether support the notion that there are persistent meiosis entry and primordial follicle replenishment in postnatal mouse ovary under physiological conditions.

Genetic tracing of *Oct4*-expressing cells suggests the presence of putative germ stem cells capable of proliferation and mitotic division

To understand whether the neo-oogenesis is based on the activities of putative germ stem cells, we examined if a portion of *EYFP*$^+$ germ cells possess the stem cell properties of long-term proliferation and mitotic division. The formation of germ cell cluster in which cells are in the process of mitotic division or had just divided and remained in close proximity to one another is often considered as a critical evidence of germ cell mitotic division. Indeed, two or more clustered *EYFP*$^+$ small germ cells were detected at each time point after tamoxifen induction (Fig. 4a–i), despite that the frequency was extremely low (<1%). Moreover, rare *EYFP*$^+$ cells were captured at metaphase or anaphase as judged by the chromosome morphology (Fig. 4j and k) at all time points examined. These observations indicate that *EYFP*-labeled putative germ stem cells undergo mitotic division to generate descendent daughter cells. Co-staining *EYFP* with *Stra8*, *Sycp3*, *Dazl* or *Oct4* documented different modes of germ stem cell division. For instance, co-staining *EYFP* with *Stra8* revealed three types of division in which both (Fig. 4d), one (Fig. 4e) or neither (Fig. 4f) daughter cells initiated *Stra8* expression. Similarly, triple staining of *EYFP*, *Oct4* and *Dazl* showed different types of germ cell mitotic division (Fig. 4g–i).

We then examined if a fragment of *EYFP*$^+$ small germ cells were capable of proliferation after long-term tracing. Adult female mice were injected

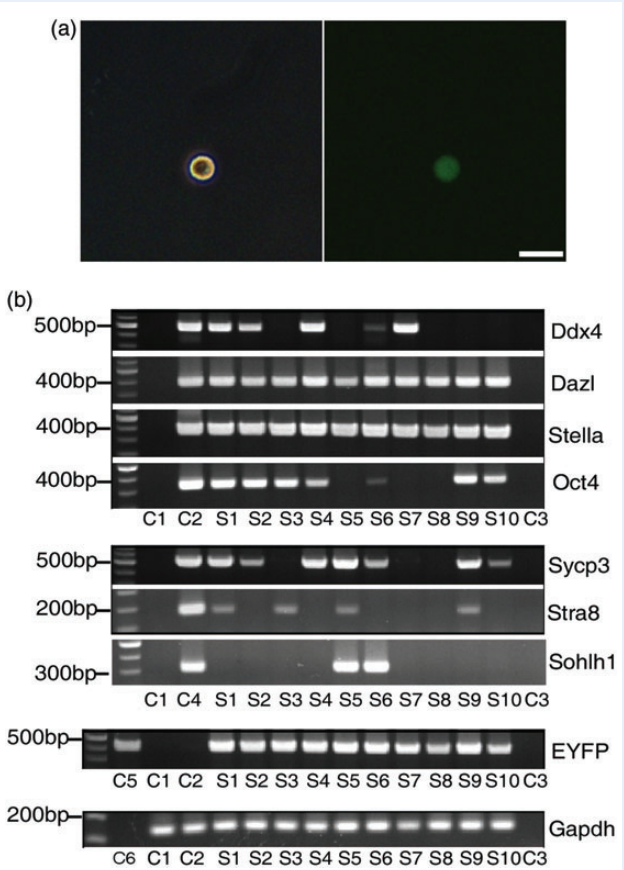

Figure 3 Genetic tracing combined with RT–PCR confirms continuous meiosis entry in postnatal mouse ovaries. Single-cell suspension was prepared from ovaries, and *EYFP*-expressing single cells were manually picked at 1 day, 3 days, 2 weeks, and 1 month post-injection of tamoxifen. (**a**) Representative *EYFP*-expressing single cell for cDNA amplification. The mRNAs expression of *Gapdh*, *EYFP*, *Stra8*, *Sycp3*, *Sohlh1*, *Oct4*, *Stella*, *Dazl* and *Ddx4* was examined by RT–PCR at all time points. The representative data collected at 1 month post-injection of tamoxifen were shown in (**b**). C1, *EYFP*-negative single cell from single-cell suspension; C2, single germinal vesicle stage oocyte from wild-type C57BL/6 mouse; C3, double-distilled H_2O; C4, mouse testicular tissues; C5, genome DNA from *R26R-EYFP*$^{Tm/Tm}$ mouse; C6, single-cell suspension medium. Samples 1–10 (S1–S10), *EYFP*-expressing single cells. Scale bar, 20 μm.

with BrdU at 4 months post-tamoxifen administration, and ovaries were collected 12 h later for BrdU incorporation assay. Consistent with the observation of persistent germ cell mitotic division, rare *EYFP*$^+$ germ cell nuclei were labeled with BrdU (*EYFP*$^+$BrdU$^+$) in comparable intensity to that observed in replicating somatic cells, indicative of the active genome DNA replication rather than DNA repair or mitochondria DNA replication (Fig. 4l and m, arrowhead). Some *EYFP*$^+$BrdU$^+$ germ cells simultaneously expressed *Stra8* protein, suggesting that these cells were undergoing pre-meiotic DNA replication (Fig. 4m, arrowhead). In contrast, other *EYFP*$^+$BrdU$^+$ germ cells were negative for *Stra8* expression, indicating that these germ cells underwent *bona fide* mitotic proliferation (Fig. 4l, arrowhead). These lines of evidence support the existence of putative ovarian germ stem cells capable of proliferation and mitotic division.

Oct4$^+$*Dazl*$^-$ small germ cells contain a population of germ stem cells in postnatal mouse ovary

Although the specific marker of ovarian germ stem cells is unclear, we postulated that they express *Oct4* but not early germ cell differentiation marker *Dazl* or *Ddx4*. To test this hypothesis, we examined the proliferative activity of the *Oct4*$^+$*Dazl*$^-$ small germ cells in juvenile female mice (3–4 weeks old) by BrdU incorporation assay as described above. Rare *Oct4*$^+$*Dazl*$^-$ small germ cell nucleus was specifically labeled with intensive BrdU signal indicative of the active genome DNA replication (Fig. 5a). A small number of *Oct4*$^+$*Dazl*$^+$ small germ cells were also positive for intensive nuclear BrdU labeling (Fig. 5b), which agreed with previous observation of BrdU$^+$*Ddx4*$^+$ cells in postnatal ovaries (Johnson *et al.*, 2004; Zou *et al.*, 2009). Given that *Dazl* expression is required for *Stra8* initiation (Baltus *et al.*, 2006; Lin *et al.*, 2008), we concluded that BrdU labeling in *Oct4*$^+$*Dazl*$^-$ germ cells reflected their *bona fide* mitotic proliferation, whereas BrdU incorporation in *Oct4*$^+$*Dazl*$^+$ cells might reflect pre-meiotic DNA replication. Indeed, some *Stra8*$^+$ germ cells were captured at pre-meiosis DNA replication (Fig. 5c). In concordance with their proliferation competence, genetic tracing revealed that *EYFP*$^+$*Oct4*$^+$*Dazl*$^-$ germ cells were small (diameters of 5–10 μm) and persisted in ovaries for 2 (4.33%, $n = 323$) and 4 months (2.13%, $n = 141$), although their number was extremely low (Fig. 5d and e). These investigations suggested that *Oct4*$^+$*Dazl*$^-$ but not *Oct4*$^+$*Dazl*$^+$ small germ cells contained a rare population of germ stem cells responsible for primordial follicle replenishment in postnatal mouse ovaries.

Dazl and *Ddx4* are early differentiation markers of germ cells. In mouse and human fetal germ cell development, *Dazl* expression is initiated earlier than *Ddx4* (Toyooka *et al.*, 2000; Anderson *et al.*, 2007; Bowles and Koopman, 2007; Guo *et al.*, 2015). In juvenile mice (3–4 weeks old), we co-immunostained ovarian sections with *Dazl* and *Ddx4* antibodies and observed that 86.7% ($n = 136$) of *Dazl*$^+$*Ddx4*$^-$ small germ cells were at 5–10 μm in diameter, whereas 81.2% ($n = 171$) of *Dazl*$^-$*Ddx4*$^+$ small germ cells had diameter of 10–15 μm. Thus, *Ddx4* is expressed in larger germ cells, suggesting that *Ddx4* expression represents more advanced stage of differentiation during the germ stem cell-based oogenesis in postnatal ovaries.

Discussion

Three recent studies utilized *in vivo* approaches to investigate the possible existence of putative ovarian germ stem cells but found no mitotically active germ stem/progenitor cells in postnatal mouse ovaries (Lei and Spradling, 2013a; Zhang *et al.*, 2012, 2014). One study used CAG promoter to drive Cre-loxp system and marked all types of cells in ovaries with *YFP* upon tamoxifen induction. By examining the numbers of *YFP*-marked primordial follicles and total primordial follicles at different time points following tamoxifen administration, they found that *YFP*-labeled primordial follicles and the total primordial follicle pool were stable as a whole. Moreover, they failed to detect the *YFP*$^+$*Ddx4*$^+$ germ cell cluster, which they considered as an unambiguous indicator of the germ stem cell mitotic activity. Based on these observations, this study concluded that there was no active germ stem cells in postnatal ovaries (Lei and Spradling, 2013a). However, the observed stability of *YFP*-marked and total primordial follicles as a whole can be alternatively explained by the replenishment of new primordial follicles from *YFP*-

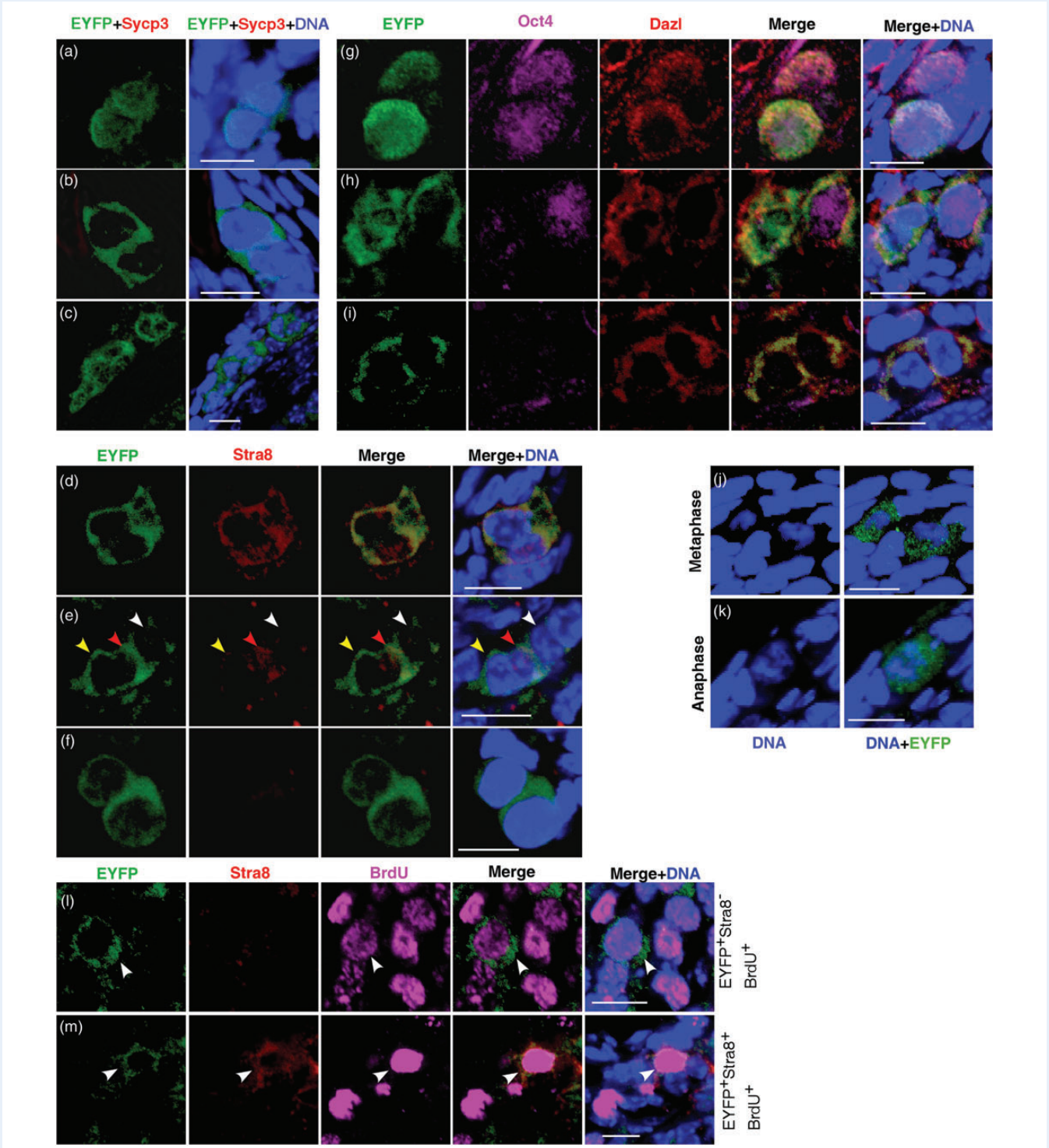

Figure 4 Genetic cell tracing reveals the continuous proliferation and mitotic division of germ cells. Representative micrographs were obtained at 4 mpi. (**a–c**) Co-staining *EYFP*$^+$ cells with antibodies to *Sycp3* documented a cluster of two or more germ cells that had just divided. None of the daughter cells initiated *Sycp3* expression. (**d–f**) Co-staining *EYFP*$^+$ cells with antibody to *Stra8* revealed three modes of germ cell division. Arrowheads in (**e**) indicated three clustered *EYFP*$^+$ germ cells. Red arrowhead, daughter cell initiating *Stra8* expression; yellow arrowhead, daughter cell negative for *Stra8*; white arrowhead, germ cell negative for *Stra8*. (**g–i**) Different modes of germ cell division revealed by immunostaining *EYFP*$^+$ germ cells with antibodies to *Oct4* and *Dazl*. *EYFP*$^+$ germ cells at metaphase (**j**) and anaphase (**k**). (**l**) After tracing for 4 months, *EYFP*$^+$ germ cell (arrowhead) undergoes *bona fide* mitotic proliferation as indicated by the active BrdU incorporation and the absence of *Stra8* expression. (**m**) After tracing for 4 months, *EYFP*$^+$ germ cell (arrowhead) undergoes pre-meiotic DNA replication as evidenced by the active BrdU incorporation and the *Stra8* expression. At each time point, ovaries from two to three animals were used for investigation. Scale bar, 10 μm.

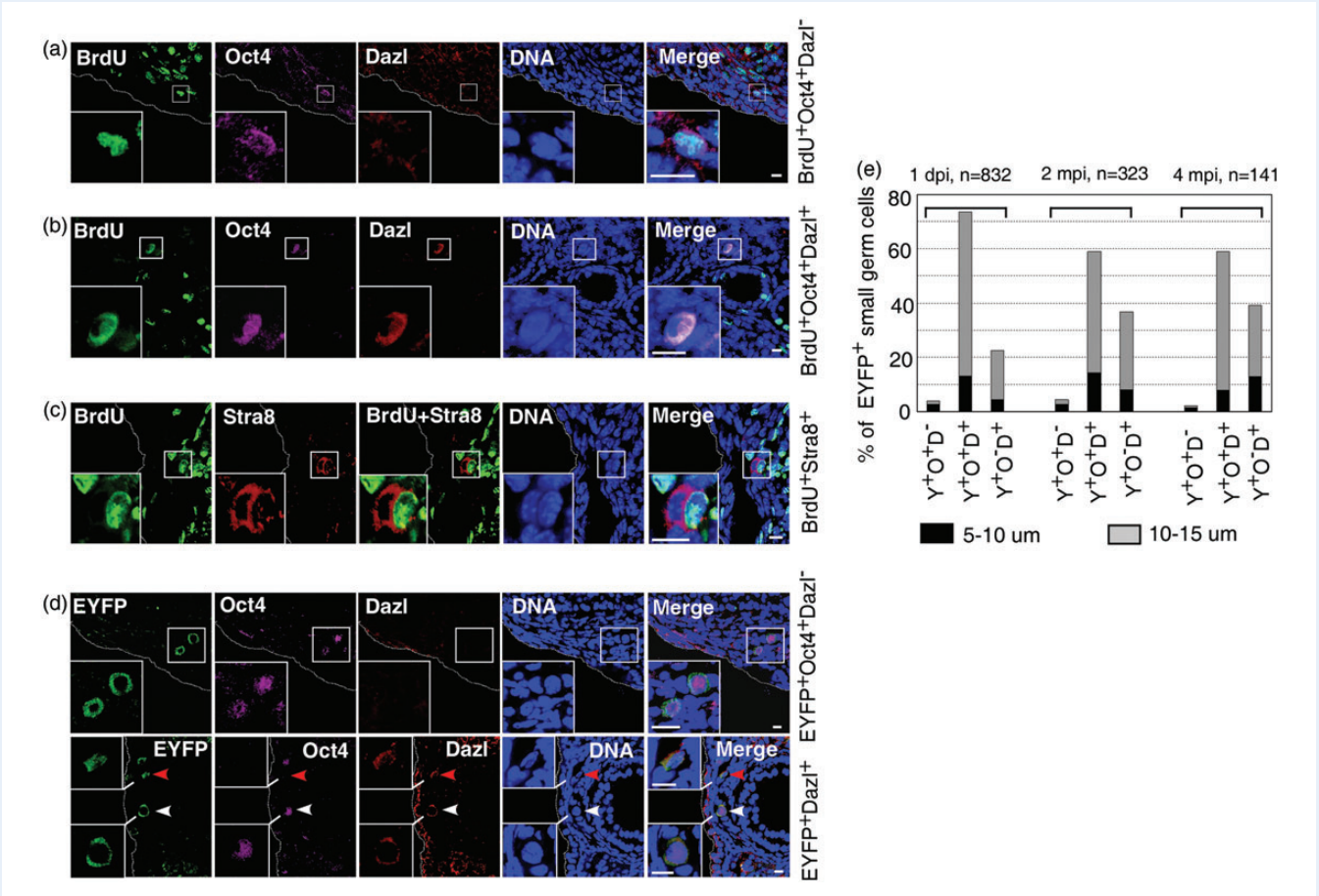

Figure 5 *Oct4*$^{+}$*Dazl*$^{-}$ cells contain a population of ovarian germ stem cells. (**a**) Rare *Oct4*$^{+}$*Dazl*$^{-}$ cells were labeled with BrdU, indicative of active genome DNA replication. (**b**) Rare *Oct4*$^{+}$*Dazl*$^{+}$ cells were also labeled with BrdU. (**c**) Germ cell undergoing meiotic DNA replication. (**d**) Genetic cell fate tracing documented the persistence of *EYFP*$^{+}$*Oct4*$^{+}$*Dazl*$^{-}$ germ cells. *EYFP*$^{+}$*Oct4*$^{+}$*Dazl*$^{+}$ (white arrowhead, $Y^{+}O^{+}D^{+}$) and *EYFP*$^{+}$*Oct4*$^{-}$*Dazl*$^{+}$ (red arrowhead, $Y^{+}O^{-}D^{+}$) germ cells were also detected, respectively. (**e**) Percentages of *EYFP*$^{+}$*Oct4*$^{+}$*Dazl*$^{-}$ ($Y^{+}O^{+}D^{-}$) small germ cells at each time point following tamoxifen injection. Insets show the higher magnification of germ cells indicated by squares. At each time point, ovaries from two to three animals were used for investigation. Scale bar, 10 μm.

marked and -unmarked putative germ stem cells. Furthermore, *Ddx4* was not expressed in putative ovarian germ stem cells, as suggested in this study and others. Rather, it is expressed in differentiated germ cells that are unable to undergo mitotic division or form germ cell clusters. The other work by Zhang *et al.* also utilized *Ddx4* to mark the putative germ stem cells and failed to detect germ stem cell activities *in vivo* (Zhang *et al.*, 2012). Thus, utilizing an inappropriate molecule, for instance *Ddx4*, to label putative germ stem cells could lead to failure in detecting ovarian germ stem cell activities as well as the wrong conclusion. The most recent work by Zhang *et al.* employed other strategies to address this question and found no neo-oogenesis *in vivo* (Zhang *et al.*, 2014). In this study, all germ cells expressing *Gdf9* were eliminated upon administration of the diphtheria toxin into the *Gdf9*-Cre;*iDTR*-targeted mutant mice. No new germ cell was regenerated after germ cell depletion. However, it is not clear if elimination of all existing germ cells in ovaries impairs the somatic cell functions and disrupts the stem cell niche necessary for stem cell regeneration. Moreover, *Sohlh1*-CreERT2;*R26R* genetic tracing was employed to label a portion of primordial follicles. The ratios of the labeled to unlabeled oocytes were compared at 2 and 8 months following tamoxifen injection at postnatal day 28. They observed similar ratios at the two time points and assumed no neo-oogenesis. However, animal age can compromise putative germ stem cell functions (Niikura *et al.*, 2009). It remains unknown if functional decline of putative ovarian germ stem cells already occurs in 9-month-old mice. More time points, e.g. 4 and 6 months post-tamoxifen injection, should be considered at this study. Furthermore, it was not clear if the unlabeled immature oocytes within primordial follicles were all counted in this study.

To find out an appropriate gene promoter to drive Cre-loxP system and to study the putative ovarian germ stem cells, we reviewed the gene expression dynamics of primordial germ cells (PGCs) in mouse and human fetal ovaries. Undifferentiated mouse PGCs abundantly express pluripotency marker *Oct4*. Between embryonic day 10.5 (E10.5) and E11.5, PGCs initiate the expression of meiotic competence marker *Dazl* (Ruggiu *et al.*, 1997; Seligman and Page, 1998), which in turn induces the expression of *Stra8* required for pre-meiotic DNA replication and subsequent events of prophase I (Baltus *et al.*, 2006; Lin *et al.*, 2008). Shortly after *Stra8* induction, *Sycp3*, required for meiotic

synaptonemal complex assembly, is strongly expressed (Feng et al., 2014). Like *Dazl*, *Ddx4* expression is initiated in differentiated PGCs at E 11.5 and increases at E 12.5 (Toyooka et al., 2000). Coinciding with the progress of germ cell differentiation, *Oct4* expression in PGCs undergoes down-regulation (Pesce et al., 1998; Bullejos and Koopman, 2004; Bowles and Koopman, 2007). Thus, gene expression shifts from *Oct4* to differentiation markers (*Dazl*, *Ddx4*, *Stra8* and *Sycp3*) along with the commitment of PGCs to meiosis. Similar gene expression dynamics was reported in the development of human fetal PGCs (Guo et al., 2015). Concordantly, we detected several types of small germ cells in the ovarian surface of juvenile mice. Those expressing *Oct4* but not differentiation marker *Dazl* or *Ddx4* have diameter of 5–10 μm and are not in follicular structure. However, the majority of small germ cells initiating the expression of differentiation markers have a diameter of 10–15 μm. These observations suggest that *Oct4* is expressed in the primitive germ cells and can be used for tracing the putative germ stem cells in postnatal mouse ovaries.

After marking the *Oct4*-expressing small germ cells with *EYFP* and tracing their fates for up to 4 months, we obtained the following findings: first, by two independent approaches of immunostaining and single-cell RT–PCR, we consistently observed the persistent mRNA and protein expression of meiosis markers *Stra8* and *Sycp3* in some *EYFP*-labeled small germ cells. Second, primordial follicles expressing *Sycp3* or *Stra8* were continuously detected by both immunostaining and single-cell RT–PCR. Because *Stra8* and *Sycp3* expression is temporary, these mRNAs or proteins cannot be carried over from the initially labeled germ cells. These results do not reconcile with the prevailing dogma. Rather, they support that meiosis entry and primordial follicle replenishment continuously take place in *EYFP*-labeled germ cells in postnatal ovaries. Third, we captured the transient processes of mitotic DNA replication as well as mitotic division of the marked germ cells at various time periods after marking, thereby providing the most direct evidence to support the neo-oogenesis in postnatal mouse ovaries under physiological conditions.

Due to the ubiquitous expression of *Oct4* in germ cells during folliculogenesis, it is not feasible to distinguish the regenerated $EYFP^+$ oocytes from pre-existing $EYFP^+$ oocytes and to verify the competence of regenerated germ cells by producing the next-generation mice. It is also difficult to assess the developmental wave of labeled germ stem cells and the contribution of germ stem cell activity to follicle pool. Nevertheless, the key features of genetic cell tracing are that it does not change the properties of the marked cell, its progeny, and its neighbors (Kretzschmar and Watt, 2012). This makes the functional test of the progeny of the marked cells not obligatory in many genetic cell lineage tracing studies (Bonaguidi et al., 2011; Schepers et al., 2012). In addition, our work proposed that $Oct4^+Dazl^-$ but not $Oct4^+Dazl^+$ or $Oct4^+Ddx4^+$ cells display *bona fide* mitotic proliferation and contain a population of germ stem cells in postnatal mouse ovary. This does not reconcile with the previous studies that reported the isolation of ovarian germ stem cells with antibody to *Ddx4* (Zou et al., 2009; White et al., 2012). Whether germ cells purified via *Ddx4* antibody are dedifferentiated during *in vitro* culture requires further investigation (Vogel, 2012). The future work should aim at identifying the specific ovarian germ stem cell marker and improving the lineage tracing system. Refined lineage tracing driven by stem-cell-specific marker gene is necessary to evaluate the ovarian germ stem cell activities under physiological conditions and their contributions to normal as well as pathological ovarian functions.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

P.Z. organized and designed the study. K.G., C.L., X.W. and D.H. performed the experiments. P.Z. and K.G. wrote the manuscript. All authors gave final approval for publication.

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Conflict of interest

None declared.

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