Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries

Hua Zhang[a,1], Wenjing Zheng[a,1], Yan Shen[a], Deepak Adhikari[a], Hiroo Ueno[b,2], and Kui Liu[a,2]

[a]Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30, Gothenburg, Sweden; and [b]Department of Stem Cell Pathology, Kansai Medical University, Moriguchi City, 570-8506, Osaka, Japan

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It has been generally accepted for more than half a century that, in most mammalian species, oocytes cannot renew themselves in postnatal or adult life, and that the number of oocytes is already fixed in fetal or neonatal ovaries. This assumption, however, has been challenged over the past decade. In this study, we have taken an endogenous genetic approach to this question and generated a multiple fluorescent *Rosa26*$^{rbw/+}$*;Ddx4-Cre* germline reporter mouse model for in vivo and in vitro tracing of the development of female germline cell lineage. Through live cell imaging and de novo folliculogenesis experiments, we show that the Ddx4-expressing cells from postnatal mouse ovaries did not enter mitosis, nor did they contribute to oocytes during de novo folliculogenesis. Our results provide evidence that supports the traditional view that no postnatal follicular renewal occurs in mammals, and no mitotically active Ddx4-expressing female germline progenitors exist in postnatal mouse ovaries.

multifluorescent tracing | oogonial stem cells | female germline stem cells

It has been generally accepted for more than half a century that in most mammalian species oocytes cannot renew themselves in postnatal or adult life, and that the number of oocytes is already fixed in fetal or neonatal ovaries (1, 2). This assumption, however, has been challenged over the past decade (3–7).

Recently, studies from primarily two research groups have put forward that functional female germline stem cells (FGSCs) and oogonial stem cells (OSCs) have been identified in adult mouse and human ovaries using antibody-based magnetic-activated cell sorting or fluorescence-activated cell sorting (4, 5, 7). Zou et al. (5) reported that the FGSCs isolated from postnatal mouse ovaries were mitotically active during in vitro culture and that the FGSCs had begun to proliferate as early as 24 h after seeding (7). Moreover, these FGSCs were reported to be stable over 68 passages and to be able to differentiate into oocytes and to regenerate functional follicles when transplanted into the ovaries of recipient female mice that had been sterilized with chemotherapy drugs (5).

The OSCs purified from adult human ovaries exhibited proliferative characteristics similar to the mouse FGSCs during in vitro culture (4). When these OSCs were injected into biopsy samples of adult human ovarian cortical tissue (where primordial follicles are often already located), and xenografted s.c. into immunodeficient female mice, regenerated primordial follicles were observed as early as 1 wk after the transplantation (4). No information, however, was provided in the study as to how far these follicles can develop (4). Whether the regenerated oocytes were meiotically competent and functional, as Oatley and Hunt timely commented (8), is also not clear.

It is noteworthy that an antibody against human DDX4 (DEAD box polypeptide 4), also known as Ddx4 or Mvh (mouse VASA homolog) in mice, was used to sort and purify the FGSCs and OSCs in both studies (4, 5). This is troublesome because human DDX4 or mouse Ddx4 is exclusively expressed in the germ cell lineage, and is known to be an intracellular cytoplasmic protein that is not expressed on the cell surface (9, 10). Another issue is the discrepancy between the proliferation and self-renewal of the FGSCs and OSCs reported in these studies (4, 5) and the finite egg production in women and in female mice (11). It is thus not clear how the purported FGSCs and OSCs contribute to the proposed follicular replenishment under physiological conditions.

As various laboratories in the field have great interest in this topic but have been unable to find functional roles for the isolated cells (6, 12), the key question is whether the cells purified using the DDX4 antibody are meiotically competent and can differentiate into functional oocytes. It is also of great importance to validate the conclusions drawn by Zou et al. (5) and White et al. (4) with an experimental approach other than an antibody-based cell purification.

In this study, we have taken an endogenous genetic approach and used *Rosa26*$^{rbw/+}$*;Ddx4-Cre* ("rbw" stands for "rainbow") mice to trace the proliferation and differentiation of Ddx4-expressing ovarian cells in vitro and in vivo. In the *Rosa26*$^{rbw/+}$*;Ddx4-Cre* mice, the endogenous *Ddx4* promoter drives the Cre recombinase in germline lineage cells and induces recombination at the *Rainbow* cassette, which is composed of four ORFs encoding four different fluorescent proteins in series (13, 14) as indicated in Fig. S1. This recombination event leads to a random switch in the expression from enhanced green fluorescent protein (EGFP) to red fluorescent protein (RFP), orange fluorescent protein (OFP), or cyan fluorescent protein (CFP) in Ddx4-expressing cells. Meanwhile, EGFP is still expressed in somatic cells (Fig. S1). Thus, the Ddx4-expressing germline lineage cells can be distinguished from the non–Ddx4-expressing somatic cells by a change in fluorescent color, as validated in Fig. S2. Our results from live cell imaging and de novo folliculogenesis experiments showed that Ddx4-expressing cells in postnatal mouse ovaries do not proliferate, and they do not contribute to oocytes during de novo folliculogenesis. Thus, in contrast to the results published by Zou et al. (5) and White et al. (4), our results show that no mitotically active Ddx4-expressing female germline progenitors exist in postnatal mouse ovaries.

Results and Discussion

Adult Mouse Ovaries Can Support but Do Not Contribute Oocytes to de Novo Folliculogenesis by Transplanted Fetal Ovarian Cells. In an effort to ascertain the existence of female germline progenitors in postnatal mouse ovaries as reported elsewhere (4, 5), we first

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[1]H.Z. and W.Z. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: kui.liu@gu.se or hueno@takii.kmu.ac.jp.

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tested the ability of adult mouse ovaries to support the formation of new follicles when provided with female primordial germ cells (PGCs) that have well-documented potential for germ cell development (15, 16). The majority of PGCs in mice migrate to the female gonad by 12.5 d post coitum (dpc) and serve as highly potent progenitors for germ cell development (15–19). These mitotically active PGCs then undergo meiosis and actively interact with support cell progenitors to form primordial follicles (18, 20).

We transplanted EGFP-expressing ovarian cells from 12.5 dpc *Rosa26*$^{rbw/+}$ fetuses into the ovaries of 2-mo-old WT C57BL/6 female mice. Four weeks after the transplantation, EGFP-positive follicles at different stages of development were observed in the ovaries of recipient females. These fluorescent follicles were found in both the cortex and medulla of the recipient ovaries, showing that the transplanted fetal ovarian cells had been randomly dispersed after injection (Fig. 1 *A*–*C*). Eight weeks after transplantation, EGFP-positive follicles could still be found in the recipient ovaries (Fig. S3 *A* and *B*). These results indicated that the adult mouse ovaries could support de novo follicular formation if potent progenitor cells that could differentiate into oocytes (such as the PGCs) were provided.

All of the follicles that developed de novo, however, consisted solely of the descendants of the originally transplanted, EGFP-expressing fetal ovarian cells, i.e., both the oocytes and the surrounding granulosa cells were EGFP positive (Fig. 1 *A*–*C* and Fig. S3 *A* and *B*). No chimeric follicles containing a combination of EGFP-positive oocytes with EGFP-negative granulosa cells or EGFP-negative oocytes with EGFP-positive granulosa cells were formed in the recipient ovaries. Thus, no cells from the adult mouse ovaries contributed to oocytes, or granulosa cells, during de novo folliculogenesis even if PGCs and active support cell progenitors were supplied. This suggests that follicular renewal is not an active physiological process, and that the purported germline progenitors (4, 5), if any, are not functionally active in normal postnatal mouse ovaries.

In addition, some EGFP-positive cells were found in the theca-interstitial layer and stromal area of several EGFP-negative follicles (Fig. 1 *A*– *C* and Fig. S3 *A* and *B*, white arrows). This suggests that the transplanted 12.5 dpc fetal ovarian cells contributed to the cell populations in these areas of the adult mouse ovary, which is in accordance with a previous report (12).

Depletion of Endogenous Follicles by Chemotherapy Drugs Does Not Stimulate de Novo Folliculogenesis with Oocytes Originating from Adult Mouse Ovaries. The chemotherapy drugs busulfan and cyclophosphamide have been widely used in the study of postnatal follicular renewal due to their ability to deplete endogenous oocytes and follicles in mouse ovaries (3, 5, 21). It has been reported that the introduction of putative germline progenitors such as bone marrow cells (21) or the purported FGSCs (5) into chemotherapy-sterilized adult mouse ovaries resulted in follicular regeneration. These putative germline progenitors were reported to recruit somatic cells of unknown origin in the recipient ovaries to form renewed follicles a few days (21) or 2 mo (5) after the transplantation. Based on our finding that no cells from adult mouse ovaries contributed to oocytes during de novo folliculogenesis after transplantation of fetal ovarian cells, we tested whether chemotherapy sterilization would stimulate adult ovarian cells to contribute oocytes to follicular regeneration.

We injected busulfan and cyclophosphamide into 2-mo-old WT female mice according to established protocols (5), and observed that almost all follicles were depleted (Fig. 1 *D* and *E*

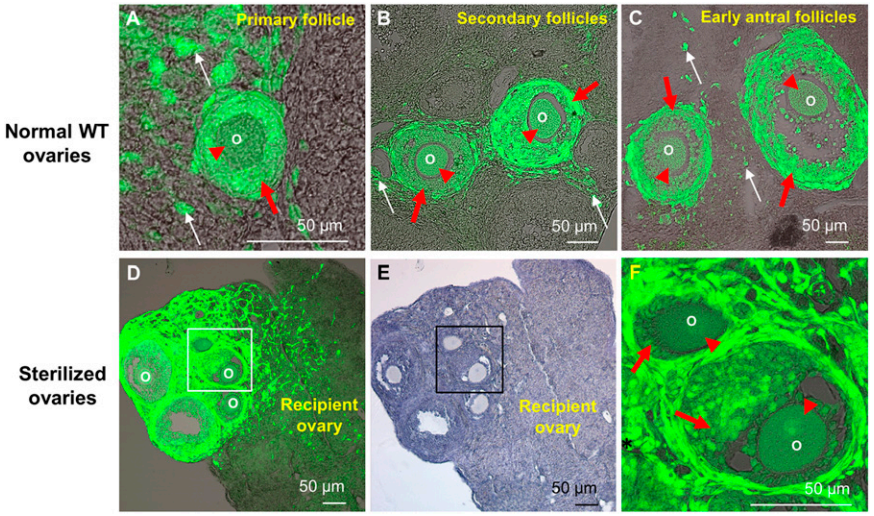

Fig. 1. Adult mouse ovaries can support, but do not contribute oocytes to, de novo folliculogenesis by transplanted fetal ovarian cells. (*A*–*C*) EGFP-expressing ovarian cells from 12.5-dpc *Rosa26*$^{rbw/+}$ fetuses were collected and dispersed through a 40-μm-pore strainer to avoid cell aggregation. Approximately 2×10^5 cells were injected into each ovary of adult WT (C57BL/6) mice at three different sites. Four weeks after the transplantation, 53 newly formed, EGFP-positive follicles were observed in the ovaries of six recipient females. Representative primary (*A*), secondary (*B*), and early antral (*C*) follicle stages are shown. All EGFP-positive follicles were composed of EGFP-positive oocytes (red arrowheads) and EGFP-positive granulosa cells (red arrows). This shows that both structures have developed from the transplanted fetal ovarian cells. Some EGFP-positive cells were seen in the theca-interstitial layer and stromal area of several EGFP-negative follicles (white arrows), but no chimeric follicles with EGFP-positive granulosa cells and EGFP-negative oocytes, and no EGFP-negative granulosa cells and EGFP-positive oocytes, were found. This suggests that adult mouse ovaries do not contribute oocytes during de novo folliculogenesis initiated by transplanted fetal ovarian cells. (*D*–*F*) Two-month-old WT females were sterilized by i.p. injection of busulfan (30 mg/kg bodyweight) and cyclophosphamide (120 mg/kg bodyweight). Two weeks later, EGFP-expressing ovarian cells from 12.5-dpc *Rosa26*$^{rbw/+}$ fetuses were injected into the sterilized ovaries. (*D*) Four weeks after the transplantation, 32 EGFP-positive follicles were observed in the ovaries of 10 recipient females. All follicles were made up of EGFP-positive oocytes and EGFP-positive granulosa cells of fetal ovarian origin, and no EGFP-negative oocytes were found. This indicates that depletion of endogenous follicles by chemotherapy drugs does not stimulate the adult mouse ovaries to contribute oocytes to de novo folliculogenesis. (*E*) Hematoxylin staining corresponding to *D*. (*F*) Magnified image of the framed area in *D*. EGFP-positive oocytes (red arrowheads) and EGFP-positive granulosa cells (red arrows) are indicated. O, oocyte.

and Fig. S4). This confirmed the sterilization effect of these chemotherapy drugs as reported previously (3, 5). Two weeks later, EGFP-expressing ovarian cells from 12.5-dpc *Rosa26*$^{rbw/+}$ fetuses were transplanted into the sterilized ovaries by multisite injection. Newly formed EGFP-positive follicles were observed in the chemotherapy-sterilized recipient ovaries 4 wk after the transplantation (Fig. 1*F*). This indicates that postnatal mouse ovaries support de novo folliculogenesis when exogenous support cell progenitors and germline progenitors are provided, and that such capability is preserved even after sterilization with chemotherapy drugs.

On the other hand, we found that all of the newly formed, EGFP-positive follicles were composed of EGFP-positive oocytes (Fig. 1*F*, red arrowheads) and EGFP-positive granulosa cells (Fig. 1*F*, red arrows), but we observed no chimeric follicles. Most of the EGFP-positive follicles had developed to advanced stages in the recipient ovaries 8 wk after the transplantation (Fig. S3 *C* and *D*), yet we still observed no chimeric follicles (Fig. S3*C*). The absence of chimeric follicles indicated that chemotherapy per se did not stimulate the follicle-depleted ovaries to contribute cells that could differentiate into oocytes, or even into granulosa cells, during de novo folliculogenesis. This finding is in accordence with previous studies with another chemotherapy drug, doxorubicin (DXR) (22, 23). In an earlier report, Kujjo et al. repeated the experiment reported by Johnson et al. (21), but were not able to observe the published spontaneous replenishment of the primordial follicle pool after treatment with DXR (22). In addition, Kerr et al. showed that postnatal folliculogenesis does not occur when the primordial follicle reserve is depleted by DXR or γ-irradiation in mice (23). In the current study, we have provided direct evidence showing that the chemotherapy-sterilized adult mouse ovaries contribute neither oocytes nor somatic cells to follicular formation even when highly potent PGCs and support cell progenitors are supplemented.

Ddx4-Expressing Cells from Postnatal Mouse Ovaries Are Mitotically Inactive. We next examined whether Ddx4-expressing cells in mouse ovaries are mitotically active. Given the facts that Ddx4 is expressed in the cytoplasm in the mouse germline lineage (9) and that an antibody against human DDX4 had been used as a key procedure to isolate the purported FGSCs and OSCs (4, 5), we have generated a *Rosa26*$^{rbw/+}$*;Ddx4-Cre* mouse model. In this genetic approach, the endogenous *Ddx4* promoter drives the expression of Cre recombinase in the Ddx4-positive germline lineage. This induces recombination at the *Rainbow* cassette, leading to a random change in expression from green EGFP to red RFP, orange OFP, or cyan CFP (Fig. S1). This method can distinguish Ddx4-expressing cells from non–Ddx4-expressing cells, both in vivo and in vitro, by directly observing changes in fluorescent color. Therefore, with this model, we can trace the development of Ddx4-expressing ovarian cells nonmanipulatively.

As shown in Fig. S2, all germline cells in the mouse ovary (Fig. S2*A*, arrows) and testis (Fig. S2*B*, arrows) were found to express RFP, OFP, or CFP, whereas gonadal somatic cells still expressed EGFP. This demonstrates the effectiveness of the *Rosa26*$^{rbw/+}$*; Ddx4-Cre* germline reporter mouse line. We are thus able to trace the possible proliferation of Ddx4-expressing germline progenitors by following the mitotic divisions of RFP-, OFP-, or CFP-positive ovarian cells using live cell imaging. Moreover, we can also determine whether the RFP-, OFP-, or CFP-positive cells can form colonies during long-term in vitro culture.

As shown in Fig. 2 and Movies S1 and S2, we followed the mitotic division of RFP-positive cells from both testes (as a positive control) and ovaries of *Rosa26*$^{rbw/+}$*;Ddx4-Cre* mice for 72 h. During this culture period, the RFP-positive cells from mouse testes divided one to three times (Fig. 2*A*, circles and squares, and Movie S1), which was in accordance with the fact that spermatogonial stem cells (SSCs) are known to be mitotically active (24). In contrast, none of the RFP-positive cells from the ovaries underwent any cell divisions (Fig. 2*B*, arrow and arrowhead, and Movie S2), and all of these cells died off over the course of the prolonged culture. These results showed that, under identical culture conditions as those reported by Zou et al. (5, 7), the Ddx4-expressing cells from postnatal mouse ovaries did not enter mitosis during the 72-h cell culture. This is in contrast to the previous report that isolated FGSCs from the DDX-4 antibody–based purification started to proliferate within 24 h after seeding (7).

To evaluate the proliferation of Ddx4-expressing cells over a prolonged period, we cultured and passed the above-mentioned primary cells, as described by Zou et al. (5, 7). We found that the cultured testicular cells, as a positive control, formed RFP-positive cell colonies after 5–8 d of in vitro culture (Fig. 3*A*, arrow), and that these colonies had the same morphology as previously

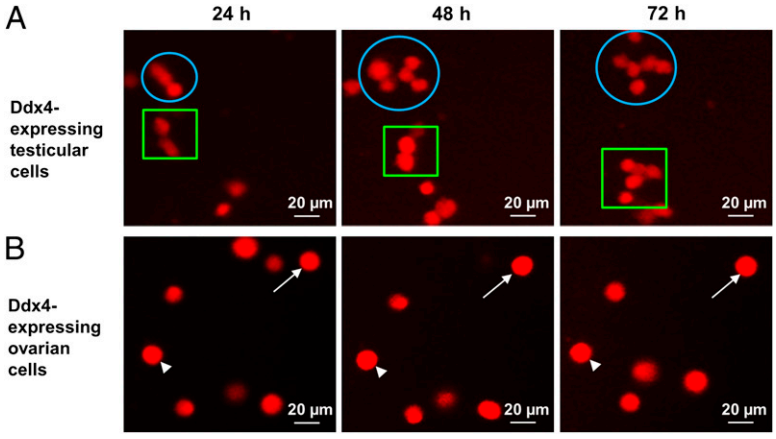

Fig. 2. Ddx4-positive cells from postnatal mouse ovaries are mitotically inactive. The gonadal cells from PD 8 or adult *Rosa26*$^{rbw/+}$*;Ddx4-Cre* male and female mice were dissociated into single cells, filtered through a 40-μm-pore cell strainer to remove large oocytes and cell aggregates, and then cultured on STO feeder cells for 12 h. The cells were then subjected to live cell imaging with an EM-CCD camera for 72 h. Photos were taken from the RFP channel every 2 h. (*A*) Representative images showing that RFP-expressing (Ddx4-positive) spermatogonial stem cells (SSCs) from postnatal testes (n = 264, 10–15 μm in size) divided one to three times (enclosed in circles and squares). (*B*) Representative images showing that none of the observed RFP-positive cells (n = 1517, 10–15 μm) from postnatal ovaries proliferated (arrow and arrowhead). Images shown are individual frames from Movies S1 and S2 (more detailed observation throughout the 72-h period is available in Movies S1 and S2).

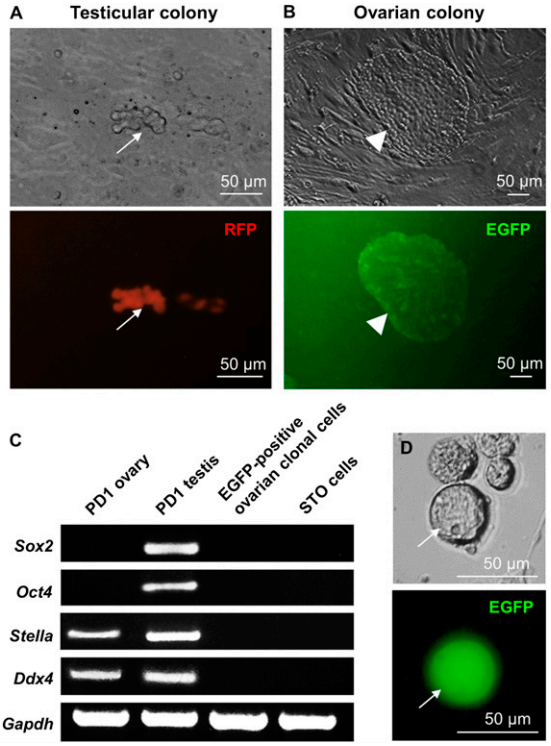

Fig. 3. Clonal cells found in long-term ovarian cell cultures are Ddx4-negative and are not germline progenitors. The gonadal cells from PD 8 or adult *Rosa26*$^{rbw/+}$*;Ddx4-Cre* male and female mice were cultured on STO feeder cells for extended periods of time. (*A*) Representative colony formed by RFP-expressing (Ddx4-positive) postnatal testicular cells after an 8-d in vitro culture (arrow). (*B*) Representative colony formed by EGFP-expressing (Ddx4-negative) postnatal ovarian cells in passage 5 (arrowhead). (*C*) EGFP-positive clonal cells found in *B* did not express pluripotent stem cell marker *Sox2* or germline markers *Oct4*, *Stella,* or *Ddx4,* as determined by semiquantitative RT-PCR. *Gapdh* was used as a control. Primers used are listed in Table S1. (*D*) Larger oocyte-like cells were observed among *Rosa26*$^{rbw/+}$*;Ddx4-Cre* postnatal ovarian cells that were cultured for 3 weeks without being passed. These larger cells are, however, EGFP positive (arrow), indicating that they are Ddx4 negative and thus are not of germline lineage. All experiments were repeated at least three times.

reported for cultured SSC colonies (25). In contrast, RFP-positive ovarian cells failed to form any colonies and were gradually degraded after several passages. These results showed that Ddx4-expressing cells from postnatal mouse ovaries were mitotically inactive.

Ddx4-negative Ovarian Clonal Cells in Culture Are Not Germline Progenitors. During the long-term cultures of *Rosa26*$^{rbw/+}$*;Ddx4-Cre* ovarian cells, some colonies were found to be formed by EGFP-positive (i.e., Ddx4-negative) ovarian cells (Fig. 3*B*, arrowheads). These colonies had a morphology similar to those reported for OSC colonies (4), and these clonal cells could be stably passed more than 10 times. Nonetheless, results from semiquantitative RT-PCR showed that these cells did not express the pluripotent stem cell marker *Sox2* (26) or the germ cell markers *Oct4* (27), *Stella* (28), or *Ddx4* (29) (Fig. 3*C*). This indicated that these EGFP-positive clonal cells were not germline cells. The absence of Ddx4 expression was in accordance with the unaltered EGFP fluorescence in these clonal cells.

To confirm these results, we tested whether these EGFP-expressing clonal cells from cultured *Rosa26*$^{rbw/+}$*;Ddx4-Cre* ovarian cells could differentiate into functional oocytes in vivo. We first transplanted these cells (at passage no. 5) into adult WT mouse ovaries through multisite injection, as described in Fig. 1. Four weeks later, we observed EGFP-expressing cells in the theca-interstitial area of some nonfluorescent follicles (Fig. 4*A*, arrows). No fluorescent oocytes or granulosa cells were ever identified (Fig. 4*A*).

This finding was further corroborated using a follicular reconstitution system (15, 30). EGFP-expressing ovarian cells from 12.5 dpc *Rosa26*$^{rbw/+}$ female mice (as a positive control) or EGFP-positive clonal cells (passage 5) derived from cultured *Rosa26*$^{rbw/+}$*;Ddx4-Cre* ovarian cells were mixed with ovarian cells from 14.5 dpc WT fetuses. The cell pellets were then transplanted under the kidney capsules of ovariectomized adult WT females. Four weeks later, the 12.5-dpc EGFP-expressing ovarian cells were found to contribute to EGFP-positive oocytes in the reconstituted follicles (Fig. S5, red arrowheads). This showed that the follicular reconstitution system worked well and supported the differentiation of germline progenitors (i.e., PGCs) into oocytes (15). On the other hand, the EGFP-positive clonal cells (as shown in Fig. 3*B*) were not found to differentiate into any oocytes in the reconstituted ovarian tissues (Fig. 4*B*). Instead these cells were found only in the theca-interstitial area of a few reconstituted nonfluorescent follicles (Fig. 4*B*, arrows).

Thus, we concluded that although the clonal cells obtained from cultures of *Rosa26*$^{rbw/+}$*;Ddx4-Cre* ovarian cells have a stem cell-like morphology (Fig. 3*B*), they are not functional female germline progenitors.

Oocyte-like Cells Observed in Long-term Cultures of Postnatal Ovarian Cells Are Not Female Germ Cells. When the above-mentioned EGFP-positive clonal cells obtained from cultures of *Rosa26*$^{rbw/+}$*;Ddx4-Cre* ovarian cells (as shown in Fig. 3*B*) were maintained for 3 wk without being passed, some larger, spherical cells were observed on top of the feeder cells (Fig. 3*D*, arrow). These cells were morphologically oocyte-like, and were similar in appearance to oocytes that have been reported to have differentiated in vitro from embryonic stem cells (31), porcine skin stem cells (32), and the purported human OSCs (4). Nevertheless, these cells were still EGFP-expressing, indicating that even if they were "oocyte-like" morphologically, they were still Ddx4-negative.

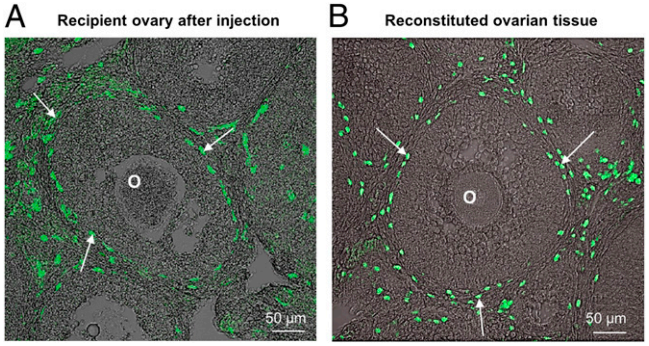

Fig. 4. Ddx4-negative ovarian clonal cells from *Rosa26*$^{rbw/+}$*;Ddx4-Cre* females cannot differentiate into oocytes or granulosa cells in vivo. (*A*) EGFP-expressing (Ddx4-negative) clonal cells from ovaries of adult *Rosa26*$^{rbw/+}$*; Ddx4-Cre* females (as described in Fig. 3*B*) were cultured for five passages and then injected into adult WT (C56BL/6) ovaries (as described in Fig. 1). Four weeks later, no fluorescent oocytes or granulosa cells were found, and EGFP-expressing cells were seen only in the theca-interstitial area of several nonfluorescent growing follicles (arrows). (*B*) In a follicular reconstitution system, the above-mentioned EGFP-expressing (Ddx4-negative) clonal cells were mixed with 14.5 dpc WT fetal ovarian cells, and the cell pellets were transplanted under the kidney capsules of bilaterally ovariectomized WT recipient females. Four weeks later, no fluorescent oocytes or granulosa cells were found in the reconstituted ovarian tissues. EGFP-positive cells were dispersed only in the theca-interstitial area of some nonfluorescent growing follicles (arrows). All experiments were repeated at least three times. O, oocyte.

In other words, these cells were not oocytes, nor were they any cells of the germline lineage. All of these cells degenerated within 3–5 d.

Conclusion

In summary, our study provides several lines of in vivo and in vitro evidence showing that the Ddx4-expressing cells from postnatal mouse ovaries did not enter mitosis, nor did they contribute to oocytes during de novo folliculogenesis. Thus, our results support the traditional view that no postnatal follicular renewal occurs in mammals, and no mitotically active Ddx4-expressing female germline progenitors exist in postnatal mouse ovaries.

Materials and Methods

Mice. The generation of *Rosa26*$^{rbw/+}$ mice was as described previously (13, 14). The *Rosa26*$^{rbw/+}$ mouse model is a multifluorescent reporter mouse line. EGFP is constitutively expressed in all cells of *Rosa26*$^{rbw/+}$ and *Rosa26*$^{rbw/rbw}$ mice. The *Ddx4-Cre* transgenic mice (33) were purchased from the Jackson Laboratory. To produce the *Rosa26*$^{rbw/+}$*;Ddx4-Cre* germline reporter mouse model, *Rosa26*$^{rbw/rbw}$ females were crossed with *Ddx4-Cre* males. In this model, the endogenous *Ddx4* promoter drives the expression of Cre recombinase in germline lineage cells, which induces recombination at the *Rainbow* cassette resulting in a random alternation of expression from green EGFP to red RFP, orange OFP, or cyan CFP. Meanwhile, EGFP is still expressed from the *Rainbow* locus in all somatic cells (Fig. S1). Ddx4-expressing germline lineage cells can thus be differentiated from the non–Ddx4-expressing cells both in vivo and in vitro by directly observing the switch in fluorescent color, as shown in Fig. S2.

WT C57BL/6 mice were purchased from Charles River Laboratories. All mice used in this study were in the C57BL/6 genetic background. All mice were housed in controlled environmental conditions with free access to water and food. Illumination was on between 0600 and 1800 h. Experimental protocols were approved by the regional ethical committee of the University of Gothenburg, Sweden.

Sterilization of Adult Mouse Ovaries by Chemotherapy Drugs. To prepare the sterilized recipient ovaries, 2-mo-old C57BL/6 female mice were injected intraperitoneally once with busulfan (30 mg/kg body weight) and cyclophosphamide (120 mg/kg body weight). The transplantation of fetal ovarian cells was performed 2 wk after the chemotherapy treatment (5).

Transplantation of 12.5-dpc Fetal Ovarian Cells into Adult Mouse Ovaries by Multisite Injection. EGFP-expressing *Rosa26*$^{rbw/+}$ fetal ovaries were collected at 12.5 dpc, and ovarian cells were prepared by a multistep digestion, as previously described (34). Briefly, the ovaries were treated with 0.125% (wt/vol) trypsin (Invitrogen) at 37 °C, neutralized with 10% (vol/vol) FBS (Gibco), and washed with PBS. After digestion, the cells were filtered through a 40-μm-pore cell strainer (BD Biosciences) to avoid any cell aggregations and then resuspended in cold DMEM-F12 (Invitrogen) with 5% (vol/vol) FBS. The transplantation procedure was performed according to Zou et al. (5). Briefly, fetal ovarian cells were injected into one of the ovaries (~0.8×10^5 cells per point, three points per ovary), and cell resuspension medium was injected into the opposite ovary as the negative control.

Preparation of Postnatal Gonadal Cells for Culture. Gonads from postnatal day (PD) 8 or adult *Rosa26*$^{rbw/+}$*;Ddx4-Cre* male and female mice were collected, cut into small pieces, and dissociated into single cells with trypsin as described above. The single-cell resuspension was filtered through a 40-μm-pore cell strainer (BD Biosciences) to remove large oocytes and cell aggregates. The same culture medium as described by Zou et al. (5, 7) was used. For long-term culture, gonadal cells were cultured on STO feeder cells in six-well plates (2×10^4 cells/well) at 37 °C in a 5% CO_2 atmosphere, as described elsewhere (5, 7). The culture medium was replaced every 2–3 d, and the cells were subcultured every 9–14 d. Images of cell colonies were taken with a Zeiss Axiovert 40 CFL inverted microscope.

Live Cell Imaging. For live cell imaging, male and female gonadal cells were first cultured on STO feeder cells in 96-well plates (2×10^4 cells/well) for 12 h for stabilization and immobilization. The 96-well plates were then transferred to the chamber of a Nikon Eclipse Ti inverted microscope for a 72-h live cell imaging. The temperature was maintained at 37 °C, and humidified air with 5% CO_2 was supplied. RFP-positive cells were monitored, and images were taken every 2 h with an EM-CCD camera (Andor).

Follicular Reconstitution and Transplantation under Mouse Kidney Capsules. Fetal ovaries were dissected from *Rosa26*$^{rbw/+}$ (12.5 dpc) and C57BL/6 (14.5 dpc) female fetuses, digested into single cells, and resuspended in standard EB medium (Millipore). The ovarian cell suspensions from a total of eight C57BL/6 fetuses was then mixed with 1×10^5 *Rosa26*$^{rbw/+}$ fetal ovarian cells (12.5 dpc) or 1×10^5 cultured postnatal ovarian cells (passage 5) along with 0.2 mg/mL phytohemagglutinin (Sigma) (30). The mixture was pelleted into grafts by centrifugation at 10,000 *g* for 1 min and cultured in standard EB medium on cell culture inserts (Millipore) at 37 °C overnight. Grafts were then transplanted under the kidney capsules of bilaterally ovariectomized C57BL/6 recipient mice.

Additional materials and methods are described in *SI Materials and Methods*.

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