Temporal Differences in Granulosa Cell Specification in the Ovary Reflect Distinct Follicle Fates in Mice

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ABSTRACT

The embryonic origins of ovarian granulosa cells have been a subject of debate for decades. By tamoxifen-induced lineage tracing of Foxl2-expressing cells, we show that descendants of the bipotential supporting cell precursors in the early gonad contribute granulosa cells to a specific population of follicles in the medulla of the ovary that begin to grow immediately after birth. These precursor cells arise from the proliferative ovarian surface epithelium and enter mitotic arrest prior to upregulating Foxl2. Granulosa cells that populate the cortical primordial follicles activated in adult life derive from the surface epithelium perinatally, and enter mitotic arrest at that stage. Ingression from the surface epithelium dropped to undetectable levels by Postnatal Day 7, when most surviving oocytes were individually encapsulated by granulosa cells. These findings add complexity to the standard model of sex determination in which the Sertoli and granulosa cells of the adult testis and ovary directly stem from the supporting cell precursors of the bipotential gonad.

INTRODUCTION

Granulosa cells play a critical role in supporting the growth and development of oocytes in immature and adult ovarian follicles. When a follicle is activated, the handful of granulosa cells surrounding the oocyte undergoes at least 10 clonal divisions to produce the more than 2000 cells of the mature antral follicle [1]. Some primordial follicles begin to grow shortly after assembly, but most lie dormant until they are activated during adult estrus cycles. The embryonic origin of granulosa cells is still not entirely clear. In mice, granulosa cells that populate the cortical primordial follicles activated in adult life derive from the surface epithelium perinatally, and enter mitotic arrest at that stage. Ingression from the surface epithelium dropped to undetectable levels by Postnatal Day 7, when most surviving oocytes were individually encapsulated by granulosa cells. These findings add complexity to the standard model of sex determination in which the Sertoli and granulosa cells of the adult testis and ovary directly stem from the supporting cell precursors of the bipotential gonad.

Foxl2, granulosa cell, mitotic arrest, ovarian surface epithelium, ovary

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results are more definitive, the fact that only a few granulosa cells were labeled left open the possibility that other cell types contribute to the granulosa cell population in the adult ovary before or after birth.

Foxl2 is a forkhead transcription factor expressed in somatic cells of the early XX gonad [13]. In goats, a female-to-male sex-reversal phenotype associated with polled intersex syndrome has been attributed to misregulation of FOXL2 expression [14, 15]. These findings led to the speculation that FOXL2 is an ovari-determining gene, parallel to SRY in males. However, in humans, mutations in FOXL2 do not lead to sex reversal, but rather to blepharophimosis-p toesis-epicanthus inversus syndrome, which results in eyelid malformations and premature ovarian failure [16]. Foxl2 is likewise not required for the initial determination of ovarian fate in mice, as null mutants do develop ovaries [13, 17], but these mutant ovaries upregulate components of the testis pathway during late embryonic development [18], and postnatal follicle activation is severely impaired [13, 17]. Moreover, deletion of Foxl2 in the adult mouse ovary led to a loss of granulosa cell identity and transdifferentiation of granulosa cells into Sertoli-like cells [19, 20].

Here we investigated the relationship between the early supporting cell lineage in the bipotential gonad and the postnatal granulosa cell population. Using the Foxl2mT[GFPCreERT2] strain (Fox2-GCE) mouse line to lineage trace the fate of the early supporting cell precursors, we show that these cells specifically contribute to the population of follicles activated immediately after birth. In contrast, those primordial follicles that grow during adult life are comprised of granulosa cells specified long after sex determination, just prior to and throughout the postnatal follicle assembly period.

MATERIALS AND METHODS

Mice

The Tg[Sry-EGFP]92Ei transgenic line, in which EGFP expression is driven by a 5' fragment of the Sry promoter, was kindly provided by K.H. Albrecht and E.M. Eicher, and maintained on the C57BL/6 background. Gt(Rosa)26Sor(cre) (R26R [21]) mice were maintained on the C57BL/6 background. The Tg(Acta2-EGFP) transgenic mouse line, in which EGFP expression is regulated by a fragment of the Acta2 promoter, was provided by L. Lessard (Children’s Hospital Medical Center, Cincinnati, OH). The Foxl2mT[GFPCreERT2] strain (Fox2-GCE), which carries a GFP-CreERT2 cassette knocked into the Foxl2 locus, was constructed by the GUDMAP consortium and maintained on a C57BL/6 background [22, 23]. Outbred CD1 animals were used to establish the time course of cell cycle arrest and for 5-bromo-2'-deoxyuridine (BrdU) and MitoTracker lineage-tracing experiments. All mice were housed in accordance with National Institutes of Health guidelines, and experiments were conducted with the approval of the Duke University Medical Center Institutional Animal Care and Use Committee.

To lineage trace Fox2-expressing cells throughout development, male mice heterozygous for the Fox2-GCE allele were crossed with females carrying the R26R reporter, and pregnant females were injected intraperitoneally with 2 mg tamoxifen (20 mg/ml) per 40 g body weight at Embryonic Day (E) 12.5 or E14.5. Embryos were allowed to develop to E14.5 or Postnatal Day (P) 7, P9, or P14 before dissection. At the indicated stages, pregnant females and pups were euthanized, and gonads were carefully removed and fixed for 30–45 min at room temperature or overnight at 4°C.

MitoTracker Labeling

Gonads were dissected from embryos/pups at stages E11.5–E14.5, P1, P3, or P7 and cultured in grooves cut in 1.5% agar blocks. The blocks were placed in 35-mm culture dishes and bathed in Dulbecco modified Eagle medium containing 10% fetal bovine serum (FBS) and 50 μg/ml ampicillin. MitoTracker Orange CMTRos (Invitrogen) was diluted in culture medium to a final concentration of 1 μM and then applied to the gonadal surface with a pipette. The dye was washed off after 30 min at 37°C, and samples were cultured for 2–96 h at 37°C with 5% CO2, and then fixed in 4% paraformaldehyde for 45 min at room temperature.

BrdU Tracing and Quantitation

Pregnant females were injected intraperitoneally with 1.5 mg BrdU (Sigma) dissolved in 7 mM NaOH/PBS at stages E11.5–E14.5. Pups were subcutaneously injected at P1 or P4 with 50 μg BrdU/g body weight. At 2 h postinjection, embryo/pups were either dissected or injected with excess thymidine (25 mg) and allowed to develop for 24–48 h (embryos) or 3–6 days (pups) before dissection. Gonads were fixed in 30% 50 mM glycerine/70% ethanol or 4% paraformaldehyde for 1 h at room temperature. Samples were washed once in PBS, treated with 2 M HCl for 30 min at room temperature, washed again, and then subjected to immunocytochemistry as described below.

To estimate the proportion of BrdU/FOXL2 double-positive cells in the total FOXL2-positive population, gonads were immunostained with antibodies against BrdU and FOXL2 and imaged at 40× magnification on an LSM710 Meta confocal microscope (Carl Zeiss, Inc.). Images (two to three per sample) were taken near the center of each gonad, with two to four gonads imaged per time point. For each image, numbers of FOXL2-positive and BrdU/FOXL2 double-positive cells were carefully counted in Adobe Photoshop and expressed as a percentage. For each injection stage, we compared the proportions of BrdU/FOXL2 double-positive cells after 24- and 48-h traces using a two-tailed Student t-test. The data are presented as the mean ± SEM.

Immunocytochemistry

Embryonic tissues were processed for whole-mount immunocytochemistry as follows. Fixed gonads were washed twice in PBS with 0.01% Tween-20 and incubated for 1 h at room temperature in a blocking solution consisting of 10% FBS, 3% bovine serum albumin (BSA), and 0.2% Triton-X-100 in PBS. Samples were then incubated with primary antibodies (listed below) in fresh blocking solution overnight at 4°C. The next day, samples were washed three times for 1 min each in a washing solution consisting of 0.1% FBS, 3% BSA, and 0.2% Triton-X-100 in PBS and then blocked for 1 h before secondary antibodies (listed below) were applied. Samples were again incubated overnight at 4°C, washed three times the next day, and mounted in DABCO. Gonads were imaged in the longitudinal plane with an LSM710 Meta confocal microscope and the affiliated Zen software (Carl Zeiss, Inc.).

Postnatal samples were transferred through a sucrose gradient, embedded in OCT, and cryosectioned at 20–25 μm. The sections were then rehydrated in PBS, blocked for 1 h, incubated with primary antibodies overnight at 4°C, washed three times for 10 min each, incubated with secondary antibodies for 1 h at room temperature, washed three more times, and then mounted in DABCO and imaged as above.

The primary antibodies used in this analysis included anti-FOXNL2 1:1,000; anti-FOXNL2 (goat anti-FOXNL2 [NB100-1277; 1:200; Novus Biologicals] or rabbit anti-FOXNL2 [1:500; a kind gift from D. Wilhelm, University of Queensland]), anti-β-galactosidase (55976; 1:10000; MP Biomedicals), anti-Ki67 (MK167) (RM-9106-S; 1:500; NeoMarkers), anti-p27 (sc-528; 1:500; Santa Cruz Biotechnology), phospho-Histone H3 (9710S; 1:500; Cell Signaling), anti-BrdU (OBT0030G; 1:200; Accurate Chemical), and anti-laminin (1:500; a kind gift from H. Erickson, Duke University). Here we investigated the relationship between the early supporting cell lineage in the bipotential gonad and the postnatal granulosa cell population. Using the Foxl2mT[GFPCreERT2] strain (Fox2-GCE) mouse line to lineage trace the fate of the early supporting cell precursors, we show that these cells specifically contribute to the population of follicles activated immediately after birth. In contrast, those primordial follicles that grow during adult life are comprised of granulosa cells specified long after sex determination, just prior to and throughout the postnatal follicle assembly period.

RESULTS

FOXL2 Marks a Granulosa Cell Precursor Lineage in the Fetal Ovary

In the mouse, FOXL2 protein expression is first apparent around E11.5 in a small number of somatic cells near the ovary/mesonephros boundary [24]. At later embryonic stages, FOXL2 is strongly expressed in the somatic cells lining germ cell nests, weakly expressed in the interstitial cells surrounding the ovarian vasculature, and excluded from the surface epithelium. In the early postnatal ovary, FOXL2 expression is apparent in both granulosa and interstitial cells, although it declines in the latter population with age [13, 25, 26].

To determine whether FOXL2 specifically marks the descendants of the bipotential supporting cell precursors in the early mouse ovary, we immunostained XX Sry-EGFP transgenic gonads with a FOXL2 antibody and assessed
colocalization at E12.5 and E13.5. Nearly all EGFP-positive cells colabeled with FOXL2 (Fig. 1), indicating that XX cells capable of activating the Sry promoter upregulate Foxl2 after committing to the ovarian pathway. However, only a subset of the FOXL2-positive cells expressed EGFP, indicating possible heterogeneity in transgene expression or in the origins or differentiation status of FOXL2-positive cells.

We then extended our analysis forward in developmental time to determine whether this early population of Foxl2-expressing cells specifically gives rise to granulosa cells in the postnatal ovary or whether it contributes to multiple cell types. To lineage trace these cells, male mice expressing GFP-CreERT2 under the endogenous Foxl2 promoter [22, 23] were crossed to females carrying the R26R reporter [21], and pregnant females were injected with tamoxifen at E12.5. Embryos were allowed to develop to E14.5 or P14 before dissection. The E14.5 XX Foxl2-GCE; R26R samples confirmed the specificity of Cre activation in FOXL2-positive cells and also revealed that the GFP-CreERT2 fusion protein was expressed in most, but not all, cells labeled by the FOXL2 antibody at E14.5 (Supplemental Fig. S1 [all Supplemental Data are available online at www.biolreprod.org]).

Ovaries from P14 Foxl2-GCE; R26R animals were stained with an antibody against β-galactosidase to visualize the postnatal distribution of lineage-traced cells (Fig. 2A). As predicted, the vast majority of cells expressing β-galactosidase were granulosa cells; a few β-galactosidase-positive cells could not be definitively identified and might have been stromal cells. Notably, β-galactosidase staining was not observed in the nascent theca cells surrounding each growing follicle (Fig. 2). Collectively, these results validate FOXL2 as a good marker of granulosa cell precursors in the embryonic ovary as well as the descendants of the bipotential supporting cell precursors (Figs. 1 and 2).

Surprisingly, lineage-traced granulosa cells were only observed in the activated (growing) follicles in the center of the ovary, not in the primordial follicles concentrated in the cortex (Fig. 2). Because our protocol targeted the first cells to express Foxl2, this result suggested a possible correlation between the stage at which a cell upregulates Foxl2 and the time at which its host follicle is activated. We hypothesized that this temporal relationship would be masked if Cre activation were delayed until later in development (e.g., E14.5). In this case, we expected that the much larger population of FOXL2-positive cells present at E14.5 would contribute to follicles in the cortex as well as the medulla. However, the distribution of lineage-traced cells in the ovaries of P9 mice exposed to tamoxifen at E14.5 (Fig. 2, B and C) was nearly indistinguishable from those injected at E12.5 (Fig. 2, A and D). Similar results were obtained with a higher dose of tamoxifen (data not shown). Therefore, the granulosa cells that populate the primordial follicles of the cortex must derive from cells that do not express the Foxl2-GCE cassette at E12.5 or E14.5. Assuming that the Foxl2-GCE cassette is activated in a random subset of cells expressing Foxl2 at E12.5–E14.5, these results suggest that granulosa cells are produced in two waves: the first corresponding to the FOXL2-positive cells derived from bipotential supporting cell precursors, which furnish growing follicles of the medullary zone, and a second pool of unknown origin that contributes to primordial follicles assembled in the cortical region of the ovary and activated throughout adult life.

**FOXL2-Positive Cells Are Arrested During Embryonic Development**

A previous study of the proliferation dynamics of somatic cells in the perinatal rat ovary distinguished two populations of ovarian granulosa cells based on location (cortex vs. medulla) and proliferation history [27]. Granulosa cells in cortical follicles were derived from cells that were actively proliferating during mid- to late gestation, whereas those in medullary follicles derived from cells that had been quiescent [27, 28]. Because our lineage-tracing results indicated that the medullary follicles of the postnatal mouse ovary derived from fetal Foxl2-expressing cells, we investigated whether these cells were quiescent during fetal life.
During Fetal Life, New FOXL2-Positive Cells Arise from Cycling Progenitors in the Surface Epithelium

A clear increase in the number of FOXL2-positive cells in the ovary was observed between E12.5 and E14.5 (Figs. 1 and 3). Because FOXL2-positive cells are mitotically arrested, this increase must be attributed to recruitment rather than proliferation. This also means that the original supporting cell precursors specified during the bipotential period cannot account for the full population of FOXL2-positive cells present in the embryo. Previous work in the testis revealed that proliferation in the surface epithelium gives rise to Sertoli cell precursors during the bipotential period [6, 33]. By E11.5, the population of Sertoli cells that will form testis cords is fully allocated, and further divisions in the surface epithelium produce interstitial cells rather than new Sertoli cells [6]. However, it remained possible that, in the ovary, the surface epithelium [34] continues to contribute to the FOXL2-positive population throughout fetal development.

To test this idea, we labeled the surfaces of XX gonads with MitoTracker, a mitochondrial dye, and then cultured the samples for 2–48 h. In E11.5 gonads cultured for 2 h, the dye was clearly restricted to the surface epithelium of the gonad (Fig. 4A). After 6 h, the label was present in more cell layers, and after 48 h, labeled cells had ingressed deep into the ovary (Fig. 4, B–D). To confirm that female supporting cell precursors derive from the surface epithelium, we labeled the surface of E11.5 Sry-EGFP ovaries with MitoTracker and cultured them for 48 h. Many EGFP-expressing cells in deeper layers of the gonad were labeled with MitoTracker, indicating that they were supporting cell precursors that had emerged from the surface epithelium of XX gonads after E11.5 (Fig. 4C). Consistent with this finding, in ovaries labeled at either E11.5 or E12.5 and cultured for 48 h, many ingressing cells expressed FOXL2 (Fig. 4, D and E). Less inward movement and rare colocalization between MitoTracker and FOXL2 were observed in cultures labeled at E14.5 (Fig. 4F). These findings indicate that bipotential supporting cell precursors and embryonic FOXL2-positive cells do indeed derive from the surface epithelium, but that this activity slows down after E12.5.

Because the surface epithelium is proliferative and FOXL2-positive cells are arrested, cycling progenitors should enter mitotic arrest after exiting the surface epithelium, but before...
embryos pulsed at E11.5 or E12.5 and allowed to develop for 48 h, a significant increase in the proportion of double-positive cells was seen (P < 0.001; Fig. 5, A and B), suggesting that there is typically a delay of more than 24 h between a progenitor cell’s last S-phase and upregulation of Foxl2. This spike was not observed in samples pulsed at E13.5 or E14.5 (Fig. 5B), suggesting that the number of new cells entering the population was proportionally much lower at these later stages. When embryos were pulsed at E15.5 or E16.5 and traced for 24–48 h, BrdU/FOXL2 colocalization was detected in the weakly FOXL2-positive interstitial cells as well as a few other FOXL2-expressing cells near the surface epithelium, indicating that these two domains were still proliferative (data not shown).

New Granulosa Cells Are Generated in the Ovarian Cortex Throughout the Follicle Assembly Period

In the outbred mouse strain used for these experiments (CD1), follicle assembly begins at birth and is nearly complete by P5. At this time, almost all surviving oocytes are individually encapsulated within primordial follicles by a dedicated set of granulosa cells [35]. Many follicles in the medullary domain are activated and begin to grow immediately upon assembly [1], whereas cortical primordial follicles remain arrested (Fig. 3). The mechanism by which some follicles are selected for activation remains unclear [36].

If the population of arrested FOXL2-positive cells specified before birth were sufficient to endow all surviving oocytes with a set of granulosa cells, no BrdU-positive granulosa cells should be detected in the primordial follicles of ovaries pulsed with BrdU during follicle assembly and traced until P7. Conversely, if a pool of cycling progenitors contributed to the granulosa cell population throughout this period, primordial follicles containing BrdU-positive granulosa cells should be easily detectable. To investigate these possibilities, we injected P1 and P4 female mice with BrdU and examined their ovaries at P7. Numerous BrdU-positive granulosa cells were detected in the primordial follicles of these samples (Fig. 6, A and B), indicating that these cells were still cycling when the BrdU was applied, and thus did not derive from the arrested FOXL2-positive population. This finding is in agreement with the earlier studies of the rat ovary [27].

We predicted that these cycling progenitors would reside in the surface epithelium, which was still proliferative at P1 (Fig. 6, C and D). BrdU staining was also observed in small clusters of CDKN1B-negative, FOXL2-negative somatic cells residing just below the surface (Fig. 6, C and D). To determine whether new granulosa cells arise from the surface epithelium during the postnatal period, we labeled P1, P3, and P7 ovaries with MitoTracker and cultured the samples for 24–96 h. In all samples, the surface epithelium was very strongly labeled after 24–48 h, but almost no inward movement was observed (Fig. 7A and data not shown). When P1 and P3 ovaries were cultured for 72–96 h, the labeled domain clearly expanded to multiple cell layers. In contrast, in ovaries labeled at P7, when follicle assembly is nearly complete, labeled cells remained almost completely confined to the surface epithelium, even after 96 h of culture (Fig. 7A).

Most ingressing cells did not express FOXL2 at these time points. However, we were able to detect several MitoTracker-labeled FOXL2-positive cells in primordial follicles (Fig. 7B) and in other less well-defined positions under the surface epithelium (Fig. 7A). To determine whether the FOXL2-negative ingressing cells were contributing to the stromal population, we used a transgenic line in which EYFP is applied, and thus did not derive from the arrested FOXL2-negative population. This finding is in agreement with the earlier studies of the rat ovary [27].

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FIG. 4. The surface epithelium is a source of new Sry-EGFP- and Foxl2-expressing cells in the fetal ovary. The ovarian surface was labeled with the cytoplasmic MitoTracker dye (MT0; magenta) at various stages of ovary development, and the samples were cultured for 2–72 h. A) Gonads from E11.5 embryos fixed after 2 h show that the dye is confined to the outermost cell layers. B) After 6 h, cell divisions in the coelomic epithelium generate labeled cells that move deeper inside the gonad. C) Ovary from an Sry-EGFP transgenic embryo labeled with MitoTracker at E11.5. Many EGFP-positive cells contained the label after 48 h of culture (overlap is white; arrowheads and inset). D) Similarly, in an E11.5 wild-type ovary cultured for 48 h, MitoTracker-labeled cells had ingressed to very deep layers, and many began to present nuclear FOXL2 staining (green; inset). E and F) Gonads labeled at E12.5 displayed fewer ingressing cells that colabeled with FOXL2 after 24–48 h. F and P) In samples labeled at E14.5, no ingression was observed after 24 h. Multiple cell layers were labeled after 48 h, but few ingressing cells were positive for FOXL2. Original magnification ×40 (A–E) or ×20 (F); bars = 20 μm.

ORIGINS OF OVARIAN GRANULOSA CELLS

upregulating Foxl2. To estimate the duration of this process, we performed a series of BrdU lineage-tracing experiments (Fig. 5A). We confirmed that FOXL2-positive cells were not in S-phase (i.e., BrdU-negative) by exposing E12.5, E13.5, and E14.5 embryos to BrdU for 2 h and then staining their gonads with antibodies against FOXL2 and BrdU (Fig. 5, A and B). In subsequent experiments, embryos were pulsed with BrdU at E11.5, E12.5, E13.5, or E14.5, injected with excess thymidine 2 h later, and then allowed to develop for 24–48 h before dissection. We then estimated the percentage of BrdU/FOXL2 double-positive cells within the total population of FOXL2-positive cells. At each stage, very few BrdU/FOXL2 double-positive cells were observed 24 h after injection. Conversely, in
FIG. 5. Cycling progenitor cells give rise to new Foxl2-expressing cells. A) Example of BrdU lineage tracing and data collection. In this series, pregnant females were injected with BrdU at E12.5, chased with thymidine 2 h later, and dissected after the indicated trace durations. Gonads were stained with antibodies against BrdU (magenta) and FOXL2 (green); white color indicates overlap between the markers. BrdU/FOXL2 double-positive cells (circles) were counted relative to the total number of FOXL2-positive cells and expressed as a percentage. Original magnification ×40; bar = 20 μm. B) Quantification of BrdU results. Embryonic stages listed above the bars indicate when BrdU was injected (n = 4–12 images per time point, from two to four independent gonad samples). Error bars indicate SEM. Embryos pulsed at E11.5 or E12.5 showed significantly higher proportions of BrdU/FOXL2 double-positive cells in 48-h traces relative to 24-h traces, but those injected at E13.5–E14.5 did not, suggesting that the number of new cells entering the population was proportionally much lower at later stages. *P < 0.001; n/s, not significant.

**DISCUSSION**

The idea that supporting cell precursors in the murine bipotential gonad give rise either to adult Sertoli or adult granulosa cells is inaccurate, at least in its simplest form. Fetal FOXL2-positive cells derived from this population do not give rise to the granulosa cells present in the adult mouse. Instead, they specifically contribute to the subset of follicles in the medulla of the ovary that are activated immediately after birth and reach antral stages before puberty (Figs. 2 and 8 and [36]). Without the required stimulation from pituitary-derived FSH, the vast majority of these follicles are destined to be lost to atresia or converted into interstitial tissue [36, 37]. In contrast, the granulosa cells that equip the cortical primordial follicles and will be activated in adult life arise at more advanced stages, as late as the end of the postnatal follicle assembly period, a few days after birth (Figs. 6–8).

Granulosa cells of dormant primordial follicles exist in stable cell cycle arrest for months, years, or even decades, waiting for the enigmatic activation signal that releases their proliferative potential and results in the final maturation of a Graafian follicle. Interestingly, the granulosa cell precursors specified early in development enter mitotic arrest concomitant with syto13. Original magnification ×20 (A and B) or ×40 (C and D); bars = 20 μm.

**FIG. 6.** New granulosa cells arise in the ovarian cortex after birth. A and B) Ovaries of P7 pups exposed to BrdU at P1 (A) or P4 (B) and stained with antibodies against BrdU (magenta) and α-laminin (green) show BrdU-positive granulosa cells inside primordial follicles (arrows, insets). As expected, granulosa cells in actively dividing medullary follicles were heavily labeled in samples pulsed at P4, but not in samples pulsed at P1. We speculate that the label was titrated out during the week-long chase in the latter case. C and D) Somatic cells in and near the surface epithelium remain proliferative after birth. Ovaries from P1 mice were injected with BrdU 2 h prior to dissection and stained with antibodies against BrdU (magenta) and CDKN1B (C) or FOXL2 (D) (green). Arrowheads point to clusters of proliferative (CDKN1B-negative, BrdU-positive) FOXL2-negative somatic cells under the surface epithelium. Nuclei (blue) were stained with syto13. Original magnification ×20 (A and B) or ×40 (C and D); bars = 20 μm.
with their embryonic specification, shortly before they upregulate Foxl2 (Figs. 3 and 5). Because they resume cycling upon follicle activation at birth (Fig. 3), their period of quiescence is relatively short. The cortical granulosa cells, in contrast, arise perinatally (E15.5–P4) from proliferating progenitors, enter mitotic arrest as they are assembled into follicles, and may remain arrested for the entirety of a female’s reproductive life.

While the granulosa cells of the medulla and cortex can be classified into two separate populations (Fig. 2 and [27]), they are likely the descendants of a single progenitor source, born at different stages of development (Fig. 8). The surface epithelium of the gonad appears to be a major, if not the only, source of granulosa cell precursors, although other potential sources cannot be wholly discounted. The first cells to emerge from this epithelium (prior to E11.5) constitute the bipotential supporting cell precursor population, competent to activate the Sry promoter and differentiate as either granulosa or Sertoli cells (Fig. 4C and [6]). In the testis, the original Sry-expressing cells and their progeny account for all of the Sertoli cells present in the adult [8]: they assemble into testis cords by E12.5, and then accommodate the growing tubules by proliferating [33] rather than recruiting new cells into the population. This does not appear to be the case in the ovary, because the number of FOXL2-positive cells increases in the absence of intrinsic proliferation (Figs. 3 and 5). New FOXL2-expressing granulosa cell precursors must therefore be recruited into the population after the bipotential period has concluded.

In accord with this observation, after sex is determined at E11.5, cells continue to drop out of the ovarian surface epithelium and, in some cases, enter mitotic arrest and upregulate Foxl2 (Fig. 4). Those that do not arrest likely contribute to the interstitial population (Supplemental Fig. S3). Surprisingly, new granulosa cells were specified from cycling progenitors in the cortex as late as P4, toward the end of the follicle assembly period (Figs. 6 and 7). In contrast, no ingression from the surface epithelium was observed in ovaries labeled at P7, even after 96 h of culture, suggesting that the role of the ovarian surface epithelium as a source of new granulosa cells normally concludes coincident with the end of the follicle assembly phase. The signals that control the temporal activity of the coelomic epithelium at fetal and neonatal stages are unknown. However, cells of the ovarian surface epithelium retain the ability to move into the ovary in adult life, as this is an important part of the postovulatory repair process in the mature animal [38].

FIG. 7. The ovarian surface epithelium gives rise to new granulosa cells during the follicle assembly period. A) Ovaries from P1, P3, and P7 pups were labeled with the cytoplasmic MitoTracker dye (MTO, magenta), cultured for 24–96 h, then fixed and stained with an antibody against FOXL2 (green, nuclear). Only the surface epithelium was labeled after 24 h, but obvious ingression of labeled cells was observed after 72–96 h in cultures started at P1 or P3, but not P7. Arrowheads point to FOXL2-positive cells labeled with MitoTracker. B) A few squamous MitoTracker-labeled FOXL2-positive cells (arrowheads) were incorporated into primordial follicles after 96 h in ovaries placed into culture at P3. Nuclei (blue) were stained with syto13. Original magnification ×20 (A) or ×40 (B); bars = 20 μm.

FIG. 8. Origins of ovarian granulosa cells. Divisions in the surface epithelium of the bipotential gonad (E11.5) produce both interstitial cells (beige) and supporting cell precursors (green) that differentiate as Sertoli cells in the presence of a Y chromosome; in XX individuals, these cells move deep into the ovary and give rise to the granulosa cells in the medullary follicles activated immediately after their assembly at birth (green). As the ovary differentiates (~E14.5), further divisions in the surface epithelium contribute more cells to the granulosa population, although they remain closer to the ovarian surface. The granulosa cells that populate the primordial follicles in the cortex (pink), which are activated in adult life, arise from the surface epithelium around birth. By P7, follicle assembly is mostly complete, and surface cells no longer move into the ovary.
In addition to their distinct cellular origins, the first wave of follicles to be activated in the ovary exhibit several properties that distinguish them from follicles activated after puberty. They progress to antral stages almost twice as fast as adult follicles [1], present distinct morphological features [28], and show altered expression of several steroidogen enzymes and steroid receptors relative to similarly sized follicles in the adult [39]. Differences between these two groups of follicles might be attributable to their different origins or to environmental factors, such as gonadotropin levels and diet, which differ in prepubertal and adult animals. Such differences are consistent with the distinct functions of follicles activated before puberty, which establish ovarian functionality at the hormonal level prior to the first LH surge [40], and those that grow during adult estrus cycles and are destined for ovulation/luteinization or atresia. In female rodents, which enter puberty around 30–40 days postpartum (e.g., [41, 42]), the first oocytes to be ovulated may actually derive from the abnormal follicles activated at birth [40]. However, in larger mammals that enter puberty many months or years after birth, the prepubertal period is characterized by the development of multiple waves of anovulatory follicles (e.g., [43, 44]); in some species, functionality of the hypothalamic-pituitary-gonadal axis is established early. FSH is produced, allowing such follicles to grow to antral stages, but the surge in luteinizing hormone required for ovulation is repressed by negative feedback from low levels of estrogen until the animal is sufficiently mature to bear young (reviewed by Rawlings et al. [45]).

The function and upstream regulators of the extended mitotic arrest of granulosa cell precursors remain uncertain. It is possible that mitotic arrest during the embryonic period prolongs and preserves the proliferative potential of granulosa cells for the time at which they will be tasked with the formidable demands of folliculogenesis. Alternatively, mitotic arrest may be required for the adoption of granulosa cell fate, or, more generally, supporting cell fate: preliminary work indicates that supporting cell precursors are arrested in both sexes during the bipotential period (data not shown). Mice carrying null mutations in the cyclin-dependent kinase inhibitors p27 or p21 do not have overt phenotypes in the embryonic ovary. However, female p27 single mutants have a larger endowment of primordial follicles at birth and exhibit premature ovarian failure and sterility after puberty, attributed to the failure of oocytes to remain meiotically arrested [32]. It is unknown whether embryonic granulosa cell precursors arrest normally in these mutants, in spite of the loss of the inhibitory factors, or whether their re-entry into cycle supports the assembly of an increased number of primordial follicles.

Mutant models that fail to arrest or prematurely resume cycling may provide insight into these issues. While FOXL2 was primarily used as a marker in the present work, it has also been linked to disruptions in cell cycle. A somatic mutation in its forkhead DNA-binding domain (C134W) is present in >95% of adult granulosa cell tumors [46, 47], and its expression is markedly reduced in the juvenile form of this disease [48]. In addition, a recent study showed that FOXL2 regulates the expression of several cell cycle inhibitors and promotes G1 arrest in cultured granulosa cell lines [49]. However, while FOXL2 may regulate the cell cycle in granulosa cells of the adult ovary, we do not attribute the embryonic mitotic arrest phenotype to the action of this protein, because cell cycle arrest seems to precede FOXL2 expression (Figs. 3 and 5 and data not shown) and is established normally in E14.5 Foxl2 mutant [13] oocytes (S. Jakob and R. Lovell-Badge, personal communication).

In conclusion, we show in the present work that the supporting cell precursors of the bipotential gonad specifically contribute to the rapidly depleted population of medullary follicles present in the prepubertal mouse. These precursors are mitotically arrested throughout fetal development, but increase in number as new cells exit the surface epithelium and upregulate Foxl2. Granulosa cells of follicles activated during adult life arise from the ovarian surface epithelium perinatally. These findings add complexity to the standard model of sex determination in which the Sertoli and granulosa cells of the adult testis and ovary directly stem from the supporting cell precursors of the bipotential gonad.

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ORIGINS OF OVARIAN GRANULOSA CELLS


