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Fate of the initial follicle pool: Empirical and mathematical evidence supporting its sufficiency for adult fertility

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Abstract

The importance of the initial follicle pool in fertility in female adult mammals has recently been debated. Utilizing a mathematical model of the dynamics of follicle progression (primordial to primary to secondary), we examined whether the initial follicle pool is sufficient for adult fertility through reproductive senescence in CD1 mice. Follicles in each stage were counted from postnatal day 6 through 12 months and data were fit to a series of first-order differential equations representing two mechanisms: an initial pool of primordial follicles as the only follicle source (fixed pool model), or an initial primordial follicle pool supplemented by germline stem cells (stem cell model). The fixed pool model fit the experimental data, accurately representing the maximum observed primary follicle number reached by 4–6 months of age. Although no germline stem cells could be identified by SSEA-1 immunostaining, the stem cell model was tested using a range of *de novo* primordial follicle production rates. The stem cell model failed to describe the observed decreases in follicles over time and did not parallel the accumulation and subsequent reduction in primary follicles during the early fertile lifespan of the mouse. Our results agree with established dogma that the initial endowment of ovarian follicles is not supplemented by an appreciable number of stem cells; rather, it is sufficient to ensure the fertility needs of the adult mouse.

Keywords: Primordial follicles; Germ cells; Germline stem cells; Follicle counts; Oocyte; SSEA-1

Introduction

A central theory in mammalian ovarian biology is that the number of follicles continually decreases with age due to both atresia and ovulation, ultimately resulting in menopause or reproductive senescence. For over 50 years, it has been widely accepted that germline stem cells in the ovary, unlike the testis, stop proliferating around the time of birth and establish an initial quota of follicles that is the only source of adult oocytes in rodents (Peters et al., 1962; Zuckerman, 1951). Hence, the processes of oogenesis and neo-folliculogenesis occur in the embryonic and neonatal animal, but not in the adult. Results reported in the companion paper indicate that follicle assembly can be regulated by activin A in the neonatal mouse, leading to an increase in primordial follicle number (Bristol-Gould, et al., this issue). Follicle loss was higher in activin-treated vs. vehicle-treated animals resulting in similar populations of follicles by the time the animals reach puberty. The follicles that are eliminated are of poor quality, leading to a quorum sensing mechanism that relies on the health of the oocyte in shaping the adult follicle pool.

To directly examine if the initial follicle pool contains a sufficient supply of oocytes for adult fertility, follicle counts from our previous study were extended through 1 year and mathematical modeling was employed to analyze the dynamics

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Table 1
Normalization factors and follicle counts

Age	n=ovaries	Normalization		Number of follicles per animal[a]		
		PF/1°	2°	PF	1°	2°
6 days	11	37.5	75	10,265±489	414±29	447±49
10 days	8	50.0	100	8662±660	567±82	984±85
19 days	6	62.5	125	5127±488	294±21	656±74
45 days	4	92.5	185	2706±387	480±54	596±90
4.5 months	4	136.0	272	1583±81	595±21	531±49
6 months	4	122.5	245	1487±109	595±23	519±55
12 months	4	115.0	230	477±145	281±67	166±41

[a]Data given as average±SEM. Abbreviations used: PF=primordial follicle, 1°=primary follicle, 2°=secondary follicle.

of follicle progression throughout the adult lifespan. This approach permitted an examination of the adequacy of the initial follicle pool to support adult fertility and the potential role of a germline stem cell population in the mouse. Both empirical and mathematical approaches were used to determine whether the initial follicle pool provides a sufficient population of germ cells necessary for fertility throughout the reproductive lifetime of the mouse.

Materials and methods

Tissue processing, follicle counting and immunohistochemistry

Follicle counts were performed on ovaries collected on postnatal days 6, 10, 19 and 45; and at 4.5 months, 6 months and 12 months using the protocols and follicle classification schemes described in the companion paper. The correction factors for determining total follicles per ovary from the average follicles per section are given in Table 1. Immunohistochemical staining for stage-specific embryonic antigen-1 (SSEA-1) was performed as described using 4- and 6-month-old mouse ovaries (Bristol-Gould, et al., this issue).

Mathematical modeling

The persistence of primordial (F_0), primary (F_1) and secondary follicles (F_2) was investigated using a mathematical model that describes primordial follicle loss and the transitions between follicle stages (depicted in Fig. 1A). These processes were modeled as first-order and equations were solved numerically using an ordinary differential equation solver in Matlab (The Mathworks, Inc, Natick, MA). In the model, k_{T0} describes the transition from the primordial to primary follicle stage, k_{L0} describes the loss of primordial follicles, k_{T1} describes the transition from the primary to secondary follicle stage, k_{T2} describes the transition from secondary to more advanced follicle stages and k_{SC}

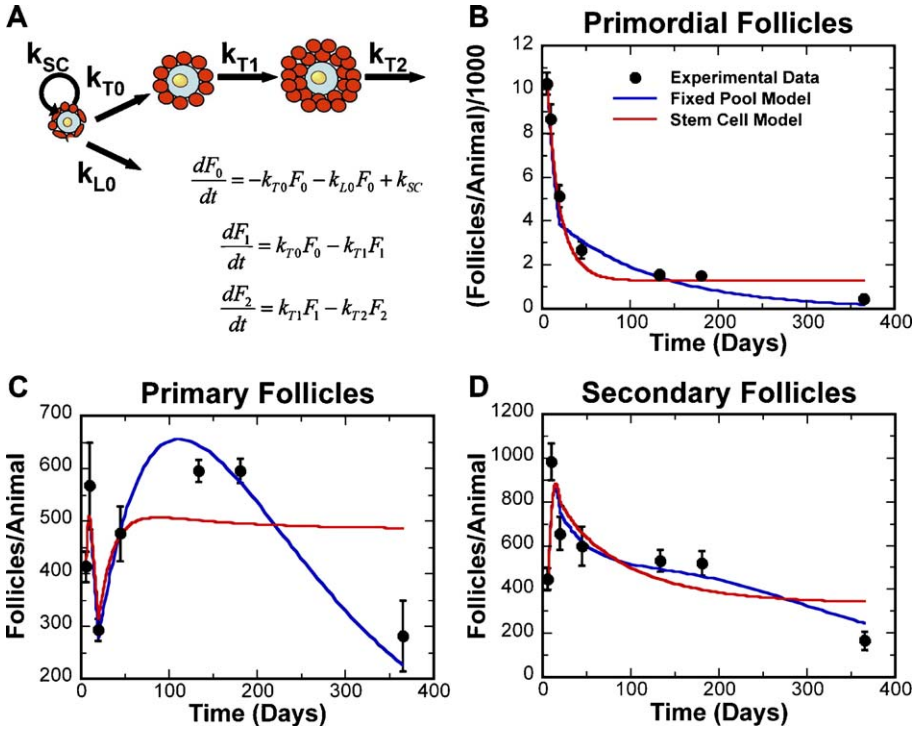

Fig. 1. Persistence of initial ovarian follicle pool. (A) Follicle dynamics were described by a series of first-order processes for loss (k_{L0}) and transition from primordial to primary (k_{T0}), primary to secondary (k_{T1}) and secondary to more mature stages (k_{T2}). In addition, the initial follicle pool was modeled as either static (fixed pool model) or as supplemented by germline stem cells (stem cell model) in a zero-order process (k_{SC}). (B–D) Follicle counts were performed on CD-1 mice from postnatal day 6 to 12 months of age and used to fit the kinetic parameters. Both models were able to capture the behavior of primordial and secondary follicles through approximately 200 days. However, the stem cell model was unable to mimic the peak in primary follicles observed at 100 days and the ultimate decline in all follicle populations (primordial, primary and secondary) beyond 200 days.

describes the generation of primordial follicles from a stem cell population. Experimental observations did not indicate a large number of morphologically unhealthy primary and secondary follicles, therefore loss of primary and secondary follicles by atresia was considered negligible. These findings are in agreement with previous data (de Bruin et al., 2002; Faddy et al., 1987; Hirshfield and Midgley, 1978). Germline stem cell generation rates were modeled as a zero-order process based on the recent report of germline stem cells in the adult mouse ovary (Johnson et al., 2004).

Results

Primordial, primary and secondary follicle counts in the adult mouse ovary

Follicles were counted in ovarian sections from mice sacrificed on postnatal days 6, 10, 19 and 45; and at 4.5, 6 and 12 months using the classifications described and the normalization factors given in Table 1. The number of primordial follicles declined sharply from day 6 to day 45, and then slowly declined through 12 months (Fig. 1B). It is difficult to directly compare follicle counts to previous reports due to both strain variation (Canning et al., 2003) and protocol variations (Myers et al., 2004). However, the trend for primordial follicle number is consistent with previous reports (Faddy et al., 1987), which reported a steep decline through day 60 and then a more gradual decline through day 100. After an initial spike, primary follicle numbers increased through approximately 6 months before declining through 12 months (Fig. 1C), whereas the number of secondary follicles remained steady before gradually declining after 6 months (Fig. 1D).

Mathematical model of the persistence of the initial follicle pool

The persistence of primordial (F_0), primary (F_1) and secondary follicles (F_2) was investigated using a mathematical model that describes primordial follicle loss and the transitions between follicle stages (primordial to primary to secondary, Fig. 1A). The persistence of follicles in CD1 mice from postnatal day 6 through 12 months was simulated based on two distinct mechanisms that may regulate the primordial follicle population: (i) a fixed initial pool of primordial follicles which serves as the only source of follicles and (ii) a stem cell population that supplements the initial primordial follicle pool with *de novo* generation of primordial follicles.

Follicle counts in CD1 mice from postnatal day 6 to 1 year were used to fit the kinetic parameters. The kinetic constants employed in simulations of both the fixed primordial follicle pool and the stem cell population models are listed in Table 2. The supply of *de novo* primordial follicles was assumed to be a zero-order process that generates 77 primordial follicles per day, as previously suggested (Johnson et al., 2004). Importantly, to fit the follicle counts, the kinetic parameters had to be solved for two separate time periods (postnatal days 6 to 20 and postnatal day 20 to 1 year). This time break is coincident with the experimentally observed spike in pituitary follicle-stimulating hormone (FSH) levels and fluctuating serum FSH levels associated with the maturation of the reproductive axis during puberty (Ackland and Schwartz, 1991; Michael et al., 1980; Rebar et al., 1981).

Table 2
Kinetic constants for models of follicle dynamics

	Fixed pool model		Stem cell model	
	Before postnatal day 20	After postnatal day 20	Before postnatal day 20	After postnatal day 20
k_{T0}	$0.024\,d^{-1}$	$0.0034\,d^{-1}$	$0.022\,d^{-1}$	$0.0034\,d^{-1}$
k_{L0}	$0.046\,d^{-1}$	$0.0053\,d^{-1}$	$0.046\,d^{-1}$	$0.056\,d^{-1}$
k_{SC}	–	–	77 F_0/d	77 F_0/d
k_{T1}	$0.40\,d^{-1}$	$0.0090\,d^{-1}$	$0.40\,d^{-1}$	$0.0090\,d^{-1}$
k_{T2}	$0.19\,d^{-1}$	$0.013\,d^{-1}$	$0.19\,d^{-1}$	$0.013\,d^{-1}$

The kinetic constants calculated for the fixed pool model after postnatal day 20 indicated that the flux from primordial to primary or primary to secondary follicle classes ranged from 3 to 6 follicles per day, consistent with previous estimates in CBA/Ca mice (Faddy et al., 1987). Three to 6 follicles per day equals 12–24 follicles per 4-day estrus cycle, and this estimate is reasonable given litter sizes from CD1 mice. These data additionally predict population half-lives of 80, 77 and 53 days for primordial, primary and secondary follicles, respectively, in the adult mouse.

The differences in the parameters between the two models were k_{L0} after postnatal day 20, the generation term, k_{SC} and k_{T0} prior to postnatal day 20 (Table 2). For the fixed pool model, the kinetic constants (k_{T0}, k_{T1} and k_{T2}) decreased by a factor ranging from 7 to 44 after postnatal day 20, indicating slower rates of transition between states. Importantly, k_{T0} decreased to a lesser extent than did k_{L0}. Therefore, the percentage of primordial follicles that transitioned to primary follicles (as opposed to being lost) increased from 34.3% to 39.1%. This increasing percentage of transitioning follicles led to the increasing number of primary follicles counted between postnatal days 19 and 134. The decreasing number of primary follicles observed after postnatal day 134 was related to the continuing decrease in the pool of primordial follicles (Fig. 1B).

In the stem cell model, k_{L0} after postnatal day 20 increased by a factor of 1.2 This increase in k_{L0} would be required to avoid an increasing number of primordial follicles that would occur due to the generation term, k_{SC}, and correlates with the unusually high rate of primordial follicle atresia suggested in the report by Johnson et al (Johnson et al., 2004). After postnatal day 20, the stem cell model predicted that only 5.7% of the primordial follicles transition to become primary follicles. Whereas this rate results in some accumulation of primary follicles, it fails to predict the maximum primary follicle number seen experimentally and predicted by the fixed pool model. Additionally, the stem cell model predicted relatively stable levels of follicles after approximately postnatal day 60, as a result of consistent introduction of *de novo* primordial follicles, a phenomena not seen experimentally (Figs. 1B–D). Other than k_{L0}, the remaining constants are

similar between the two models, which reflects a similar flux of follicles through the different stages, consistent with the experimental data.

SSEA-1 staining fails to identify germline stem cells in the adult mouse ovary

Due to potential strain variation, the predicted value of k_{SC} equal to 77 *de novo* follicles per day may not be accurate for CD1 mice (Byskov et al., 2005; Canning et al., 2003; Faddy et al., 1983; Jones and Krohn, 1961). In an attempt to refine the model parameters for germline stem cell generation by counting germ cells over time, adult ovaries were sectioned and stained for SSEA-1 a carbohydrate epitope expressed on the surface of primordial germ cells (Marani et al., 1986). This marker was utilized in a report to isolate and immunolocalize putative germline stem cells (Johnson et al., 2005). Adult reproductive tissue stained for SSEA-1, but protein was located in blood vessel lumens within the oviduct (Fig. 2A) and within the vasculature of the theca cell layers between large follicles (Figs. 2B–D). Significant staining was also observed in corpora lutea and the antral fluid of large follicles (Figs. 2B and C). This monoclonal antibody produced moderate levels of background staining, and no blocking peptides were available to test specificity. Nevertheless, because evidence of exclusive germline stem cells was not observed in the adult ovary using this marker and staining appeared to be specific for cells within the blood, this marker is likely to be detected in other organs (Telfer et al., 2005). These data are consistent with the view that SSEA-1 may be a carbohydrate epitope produced by stem cells found in the blood and not specific to stem cells of the germline (Telfer et al., 2005).

Impact of germline stem cell generation rate in the mathematical model

Although SSEA-1 staining was unable to confirm the existence of germline stem cells for quantification, the stem cell modeling approach was not limited to examining only SSEA-1-positive germline stem cell populations. To indirectly examine the impact of *de novo* follicle production on follicle dynamics, k_{SC} was varied from 5 to 100 follicles per day (Fig. 3). These simulations indicated that only low rates of generation are consistent with experimental observations (less than 10 *de novo* follicles per day). Even at these low rates, however, the generation term predicts an accumulation of primordial and secondary follicles after 6 months that is not supported by experimental data (Figs. 3A and C). Additionally, increasing k_{SC} above 25 *de novo* follicles per day failed to model the maximum primary follicle counts seen experimentally by approximately day 100 (Fig. 3B).

Discussion

The dynamic and potent regulation of the initial follicle pool by activin A (see companion paper; Bristol-Gould et al., this issue) led us to develop a mathematical model to examine the importance and fate of the initial follicle pool as it relates to the rate of follicle loss in the aging ovary and to the timing of follicular exhaustion and reproductive senescence. The dynamics of follicle populations from postnatal day 6 through 12 months were simulated according to two distinct models, which allowed us to examine if the initial population is sufficient to support the observed number of follicles present during adulthood and to quantitatively examine how a germline

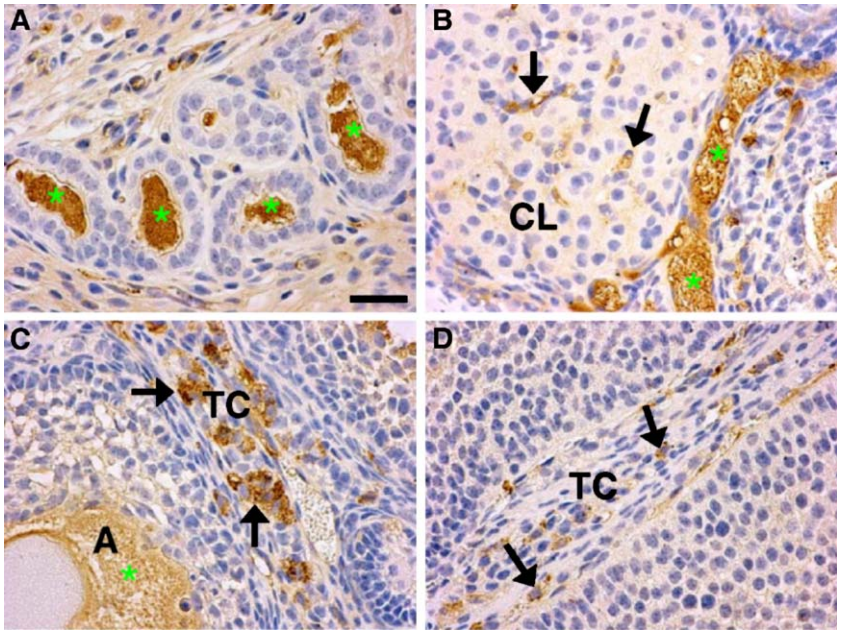

Fig. 2. SSEA-1 immunohistochemistry. (A) Adult tissue stained for SSEA-1 showing densely immunoreactive blood vessel lumens within the oviduct. (B) SSEA-1 staining within a corpus luteum (CL) and the theca cell layer between a CL and a follicle. (C) SSEA-1 staining within antral fluid of an antral follicle. (C and D) Positive SSEA-1 staining within the theca cell layers between large follicles. *Staining within large fluid filled areas (blood vessels or antral fluid). Black arrows indicate intense staining within the theca cell layers or in CLs (areas of vasculature in the adult ovary). A—antrum, CL—corpora lutea, TC—theca cell layer. Scale bar: 50 μm (A–D).

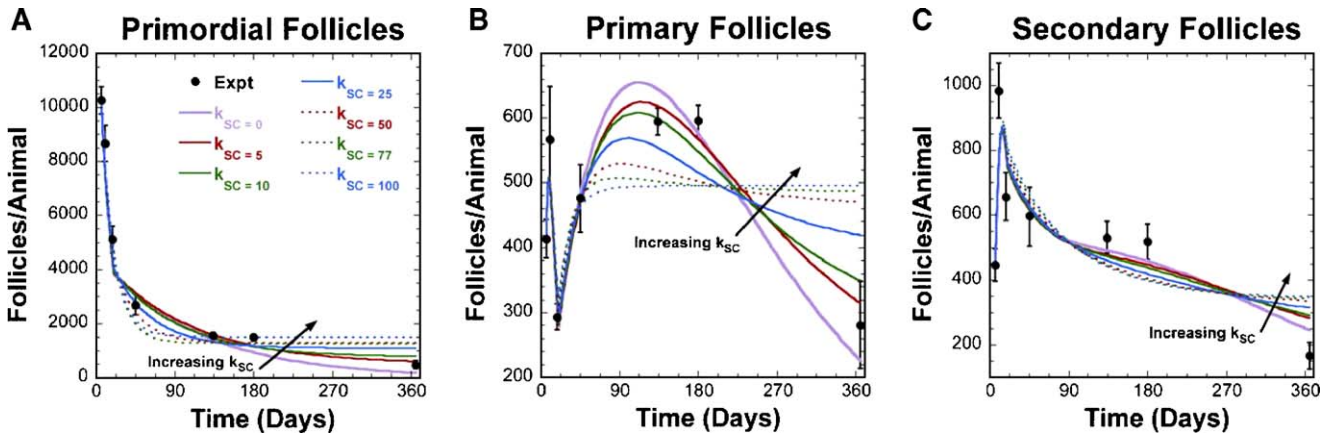

Fig. 3. Adjusting the stem cell generation rate. Varying k_{SC} from 0 to 100 *de novo* follicles per day resulted in an accumulation of primordial (A) and secondary follicles (C) that did not reflect the experimental data or that derived using the fixed pool model ($k_{SC}=0$). High k_{SC} values (>10 follicles/day) failed to describe the increase and subsequent reduction in primary follicles seen experimentally by 100 days (B).

stem cell population would impact follicle dynamics. The fixed pool model began with the assumption of a finite initial follicle pool established at birth, and with this constraint, this model was able to recapitulate the experimentally observed decrease in primordial, primary and secondary follicle numbers seen over time. Specifically, the fixed pool model fit experimental data collected during the young adult fertile days of mouse life, accurately describing the maximum primary follicle number measured at 4–6 months.

Although the mathematical modeling results suggest that the initial primordial follicle pool is sufficient for adult fertility, it has been suggested that *de novo* production of primordial follicles is required to support normal fertility in the mouse (Johnson et al., 2004, 2005). To test whether any germline stem cells could be identified in adult ovaries, adult tissue was stained with the SSEA-1 stem cell marker to count the number of stem cells at each time interval with the intent of adding these numbers into the mathematical model of follicle disposition. However, no germline stem cells were identified within the adult mouse ovary using this proposed germline stem cell marker. Instead, immunopositive areas were found within antral fluid, the vascular bed of the theca cell layer, CLs and within endothelial cell lined blood vessels. As concluded previously, this marker (and others Johnson et al. used to distinguish germ cells from somatic cells) may not be exclusive to germ cells. Indeed, several of the markers used have been localized in various organs, including brain and bone. Thus, these markers may be more accurately classified as stem cell, rather than germ cell, specific (Telfer et al., 2005).

Despite the lack of SSEA-1-positive cells, the stem cell model was modified to test the possibility of primordial follicle replenishment by incorporating the previously suggested 77 *de novo* follicles per day into the model (Johnson et al., 2004). With the addition of stem cells, the model failed to describe the experimentally observed decreases in primordial, primary and secondary follicle classes past postnatal day 200. Unlike the fixed pool model, the stem cell model did not reflect the accumulation and subsequent reduction in primary follicle numbers during the early fertile lifespan of the mouse. One limitation of this model is that it assumed a constant generation rate of primordial follicles (Johnson et al., 2004). A decreasing generation rate of primordial follicles with increasing age could allow for a lower rate of primordial follicle atresia, which may alter the predicted number of primary follicles. Indeed, germline stem cells could lose their ability to multiply as an animal ages, thereby explaining reduced fecundity with increasing age (Byskov et al., 2005). However, in the absence of experimental data measuring the number of germline stem cells over time, we were unable to further refine the estimate for the rate constant of primordial follicle generation from germline stem cells.

As the proposed 77 *de novo* follicles per day did not fit the experimental data, *de novo* follicle generation rates from 5 to 100 follicles per day were examined. These simulations indicated that only low rates of generation are potentially consistent with experimental observations (less than 10 *de novo* follicles per day). These potential generation rates appear insignificant in relation to the size of the follicle pool and are within the error of follicle counting methods. Lastly, even at a generation rate of less than 10 *de novo* follicles per day, proof of a *de novo* functional oocyte initiating meiotic prophase and becoming enclosed as a diplotene oocyte has yet to be demonstrated in the adult mouse (Byskov et al., 2005). Our results agree with the concept that the initial endowment of ovarian follicles is not supplemented by an appreciable number of stem cells; rather, the initial pool is sufficient to ensure the fertility needs of the adult mouse. In combination with the results in the companion paper (Bristol-Gould, et al., this issue), we conclude that the reduction of follicle number in the mouse ovary is not a stochastic process but is regulated both prior to puberty and again in the adult animal.

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