Classification of small type B/C follicles as primordial follicles in mature rats

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In the present study, follicles were classified according to the morphology of their granulosa cells. Type B follicles contained only flattened granulosa cells; type B/C follicles had a mixture of flattened and cuboidal granulosa cells in a single layer, and type C follicles had a single layer of cuboidal granulosa cells. The primary objectives of the study were to determine whether 5-bromo-2-deoxyuridine incorporation into type B/C follicles was a marker for initiation of growth and how long type B/C follicles could remain at the same stage before transformation to type C follicles. Female Holtzman rats received bromo-deoxyuridine for 7 days. After the infusion (day minipumps were removed = day 0), rats were ovariectomized on days 0 (n = 9), 30 (n = 8), 90 (n = 8) and 150 (n = 9). The numbers of type B, B/C and C follicles within one ovary were determined using modified fractionator counting. Analysis over all times demonstrated that there were more (P < 0.0001) type B/C (941 ± 61 per ovary) than type C (140 ± 18 per ovary) or type B (159 ± 19 per ovary) follicles. The numbers of type B and type C follicles did not differ from each other at any time. Only one of 34 rats evaluated had bromo-deoxyuridine-labelled type B follicles. On day 150, 57% of the bromo-deoxyuridine-labelled type B/C follicles remained from day 0. It is concluded that (1) DNA synthesis in granulosa cells of type B/C follicles is not a reliable indicator of impending growth; and (2) type B and type B/C follicles are both components of the pool of primordial follicles.

Arguments against type B/C follicles being activated and growing is that significant growth does not occur until after all the granulosa cells become cuboidal (Gougeon and Chainy, 1987). In addition, the vast majority of follicles in cattle ovaries are type B/C follicles (van Wezel and Rodgers, 1996). If type B/C follicles make up the majority of follicles in the ovary of young animals, and if they are continuously growing, it seems unlikely that reproductive life could last as long as it does (van Wezel and Rodgers, 1996).

The primary objectives of the present experiment were to determine whether type B/C follicles comprise a component of the pool of primordial follicles and the period that they can remain arrested at this stage of development.

Materials and Methods

Female Holtzman rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained under controlled conditions of temperature (23°C) and lighting (12 h light:12 h dark; lights on at 06:00 h). Vaginal smears were performed each day and a minimum of two consecutive normal-length (4–5 day) oestrous cycles was required before implanting rats with bromo-deoxyuridine (BrdU)-containing minipumps. BrdU was dissolved in 0.9% saline to a concentration of 20 mg ml⁻¹. Osmotic minipumps (Model 2ML1; Alza Corporation, Palo Alto, CA) were filled completely with the BrdU solution and incubated in...
physiological saline for 2 h at 37°C, to prime the minipumps before they were implanted in the rats.

Rats, 62–116 days of age, were anaesthetized with ether and the minipumps were inserted subcutaneously between the shoulder blades. The small incision was closed with surgical staples. Minipumps were removed from rats after 7 days of infusion. The time of minipump removal was designated as day 0. On day 0, rats were divided equally within age into four groups. Equalizing ages across groups prevented any potentially confounding effects due to the decrease in the number of follicles that occurs with increasing age (Arai, 1920). Ovaries were removed from groups 1, 2, 3 and 4 on days 0, 30, 90 and 150, respectively.

Once BrdU is incorporated into DNA, it remains stainable in the cells for at least 350 days, providing no additional DNA synthesis occurs (Meredith et al., 1997). Therefore, if the same number of type B/C follicles were labelled with BrdU on day 150 as at day 0, this would be an indication that none of the BrdU-labelled type B/C follicles had developed to type C (oocytes surrounded by a single layer of only cuboidal granulosa cells) or beyond. This is because type B follicles seldom incorporate BrdU (Gaytan et al., 1996; Meredith et al., 1999), so there is no source of BrdU-labelled follicles to enter the pool of B/C follicles. Conversely, if there were no BrdU-labelled type B/C follicles on day 150, this would be an indication that type B/C follicles developed through this classification in fewer than 5 months.

Ovariectomy was performed at the appropriate times after removal of the minipumps. Ovaries were fixed for 2 h in Carnoy’s solution and stored in 70% alcohol until processing. At processing, ovaries were mounted in paraffin wax and sectioned serially at a thickness of 7 μm. Type B follicles cannot be distinguished accurately from type B/C follicles when they are evaluated in single histological sections (Meredith et al., 1999). Therefore, it was critical that these two groups of follicles be correctly identified and accurately counted in the present experiment. Either fractionator or disector counting can be used for determining number of objects within tissue sections (Gundersen, 1986). An example of disector counting of follicles has been described by Miller et al. (1997). In the present study, the fractionator method of counting was used for the current experiment because with this method it is not necessary to determine section thickness.

Information regarding the validation and theoretical considerations of the fractionator procedure have been reported by Gundersen (1986) and will not be discussed here. The fractionator requires that two adjacent sections be used for counting, rather than single sections. Follicles are counted when the unique identifier (the nucleus in the present experiment) appears in the first section, but not in the following section. In the present study, the fractionator method was modified where three rather than two adjacent sections were evaluated. This was necessary to classify the follicles accurately as type B or type B/C (Meredith et al., 1999). Since three sections were saved, sections 2 and 3 in the series were used for the purposes of counting with the fractionator method (Fig. 1).

In the present study, after a random start between 1 and 40, every fortieth ovarian section was counted and classified using the adjacent sections (Fig. 1). For example, if the random number chosen was 21, then the first series of sections saved would be 20, 21 and 22; the second series saved would be 60, 61 and 62 (40 sections apart); and the third series would be sections 100, 101 and 102. This process continued through the ovary until all the appropriate sections were saved. The saved sections were then processed for immunohistochemistry as described by Meredith and Doolin (1997).

A drawing tube was attached to the microscope to follow individual follicles through the three adjacent sections, particularly in sections with a high density of small follicles. The drawing tube allowed a grid on the table to be visible in the ovarian section being examined in the microscope. Section 2 in the series of three was examined first and a reference point and all follicles were drawn on an acetate located at a fixed position on the table. Section 3 was then evaluated and the reference points were aligned so that the follicle position could be compared with the location of the follicles drawn on the acetate from section 2. If follicles overlapped significantly in sections 2 and 3, they were not counted. Section 1 was then evaluated to verify the correct classification of any counted follicles from section 2. This method for comparing follicles on adjacent sections has been described in detail by Miller et al. (1997).

Follicle classification in the present experiment is similar to that reported by Gougeon and Chainy (1987). In this classification, oocytes surrounded by: (1) only flattened granulosa cells are type B follicles; (2) a mixture of flattened and cuboidal granulosa cells are type B/C follicles; and (3) a single layer containing only cuboidal granulosa cells are type C follicles. This method for classification was used in an attempt to prevent confusion regarding the term primordial follicles. When primordial follicles are used as a term in the present manuscript it is meant to denote those follicles that provide the lifetime supply of follicles for growth. The number of type B, B/C and C follicles showing BrdU uptake in at least one granulosa cell was determined. Antral follicles
were examined for the presence of BrdU label, but were not quantified.

BrdU-labelled follicles in ovaries of day 0 and day 150 rats also were evaluated: (1) the number of granulosa cells per follicle containing BrdU-label; (2) the total number of cuboidal cells within the labelled follicle; and (3) the number of cuboidal cells per follicle that were labelled. This information was used as an internal control, because of reports of the long-term detrimental effects of BrdU (Novtna et al., 1994). However, these detrimental effects are found predominantly in cells that have divided numerous times after BrdU incorporation into the cell (Ackland et al., 1988; Raffel et al., 1989; Novtna et al., 1994). It is suggested that, if BrdU labelled a minimum number of granulosa cells (for example, one granulosa cell expressing BrdU label in a follicle), and many of the unlabelled cells of the same follicle were cuboidal (one or two cuboidal granulosa cells unlabelled), then any problem with the BrdU should not be a significant problem during mitosis, because the unlabelled cells would not be damaged and should undergo mitosis normally.

Two technicians evaluated the follicles for this experiment. Ovaries were divided equally between counters, within each time category, so that any differences in numbers owing to technician could be determined statistically.

Data were analysed as a one-way ANOVA using the general linear model (GLM) procedure of SAS. A square root transformation was performed on all data before analysis. Least significant differences were used to separate individual treatment means (SAS, 1989). All data shown are least square means ± SEM and are expressed on a per ovary basis. Differences in means were considered significant when $P < 0.05$.

**Results**

Analysis performed across all times demonstrated that the two technicians did not differ ($P > 0.05$) in the number of type B follicles or type C follicles counted. However, technician 1 obtained higher counts (1069 ± 80; $P = 0.03$) than technician 2 (813 ± 80) for type B/C follicles. This had little impact on the interpretation of the data because of the large differences in number among the three follicle classifications. Type B/C follicles always comprised 72–80% of the total population of the follicles counted in the present study (Type B/C divided by (type B + type B/C + type C follicles)), and 86 ± 3% when type C follicles were deleted from the calculations (Fig. 2). Time after removal of the BrdU-containing minipumps had no significant effect on the percentages of follicles within each category (Fig. 2). There were no differences between the number of type B and type C follicles at any time. The number of type B/C, but not type B or type C follicles decreased ($P = 0.05$) with increasing time from minipump removal (Fig. 3).

Small intestine was used as a positive control for the assay procedure (Fig. 4a). BrdU labelling is clearly visible on a type C follicle and a large follicle from an ovary of a day 0 rat (Fig. 4b). All large follicles were heavily labelled on day 0 as expected after 7 days of BrdU infusion. Follicles from day 90 rats are shown (Fig. 4c,d) and some type B/C follicle were still expressing BrdU label (Fig. 4c). Unlabelled antral follicles and type B follicles were also observed adjacent to BrdU-labelled type B/C follicles (Fig. 4d). On days 90 and 150, no BrdU-labelled antral follicles were observed. This was expected because the BrdU label on a granulosa cell from a type B/C follicle would not be visible after undergoing mitosis several times. The percentage of type B, type B/C and type C follicles that contained at least one
granulosa cell labelled with BrdU are shown (Fig. 5). Only one of the 34 rats evaluated had BrdU-labelled type B follicles (represented by the 2% shown at time 0 in Fig. 5). The majority of type C follicles were labelled at time 0, but the percentage with label decreased ($P = 0.02$) with increasing time from removal of the BrdU-containing minipumps. The percentage of type B/C follicles containing BrdU was lower ($P < 0.05$) on days 90 and 150 than it was on days 0 and 30. Calculations based on these data reveal that 57% of the follicles originally labelled as type B/C remained as type B/C for the duration of the study.

Analysis of the day 0 and day 150 BrdU-labelled type B/C follicle revealed no differences ($P > 0.05$) due to time in the mean number of granulosa cells containing BrdU label (1.3 ± 0.2 and 1.4 ± 0.4 for days 0 and 150, respectively).

There were no differences in the total number of cuboidal cells in the BrdU-labelled follicles (3.1 ± 0.7 and 3.1 ± 0.7 for days 0 and 150, respectively) or in the number of cuboidal cells that contained BrdU label in the BrdU-labelled follicles (1.1 ± 0.3 and 1.0 ± 0.3 for days 0 and 150, respectively).

**Discussion**

The present study showed that the majority of type B/C follicles remain at the same stage for up to 5 months, and so type B/C follicles should be considered a component of the pool of primordial follicles. Therefore, the pool of primordial follicles includes two histologically distinct populations of follicles in mature rats. This is in contrast to the situation in
immature rats in which this group of follicles appears to grow continually, showing a high proportion that incorporate [\(^{3}H\)]thymidine (Hirshfield and DeSanti, 1995). The proportion of labelled follicles continues to decrease until day 30, at which time these type B/C follicles begin to develop in the pattern observed in mature rats.

The results of the present paper differ from those reported by Hirshfield (1989) for rats and Mariana and de Pol (1986) for rabbits. One of the objectives in these studies was the determination of the doubling time of these small follicles. Hirshfield (1989) estimated that, in rats, it may take more than a month for these small follicles to grow to more than 20 granulosa cells. However, this did not mean that all cells that were labelled at the time of [\(^{3}H\)]thymidine infusion continued to grow, even at this slow pace. If all of these follicles continued to grow at the same rate then, by 5 months, none of the type B/C follicles would be labelled, since these would have developed past the 20 granulosa cell stage (Hirshfield, 1989). The present study demonstrates that some of the labelled type B/C follicles continue to grow, but many appear to suspend their growth process with their granulosa cells exiting from the cell cycle.

Vaginal opening of Holtzman rats used in the present study normally occurs between 32 and 42 days of age. Reproductive senescence is progressive, with these rats showing a 67% reduction in the number of oestrous cycles of normal duration by 380 days of age, although they continue to cycle and can still conceive (S. Meredith, unpublished). Therefore, optimal reproductive lifespan is slightly greater than 1 year in these rats. This observation is in reasonable agreement with studies reported in the genetically similar Sprague–Dawley rats (for review, see vom Saal and Finch, 1988). Use of the values of 32 days for age at vaginal opening and 380 days for age at reproductive senescence would mean that 57% of BrdU-labelled type B/C follicles remained as type B/C follicles for at least 43% of the optimal reproductive life of these rats.

The only category of follicles that decreased significantly with increasing age was type B/C follicles. Therefore, it seems feasible that most of the type B follicles transformed into type B/C follicles early in life to provide the major source from which growing follicles are chosen. The transformation of type B follicles into type B/C follicles may represent activation. These follicles would then be prevented from growing by the inhibitory actions of local regulators, such as activin A, as has been suggested by Mizunuma et al. (1999). These authors demonstrated that follicles with multiple layers of granulosa cells and coincubated with smaller follicles arrested the development of the smaller follicles, yet the growth-arrested follicles remained healthy. Activin A appeared to be the primary inhibitor produced by the follicles with multiple layers of cells (Mizunuma et al., 1999). These data are in agreement with results obtained after hypophysectomy (Edwards et al., 1977). Hypophysectomy of mice increases the rate of growth into the category of follicles containing two layers of granulosa cells, but decreases the number of larger follicles. The increased movement into this category is likely the result of the loss of inhibition by substances from larger follicles, due to their decrease in numbers, as was suggested by Edwards et al. (1977). Activin A may be one of these substances. Although the study by Mizunuma et al. (1999) did not show a direct inhibitory influence on type B/C follicles, inhibition of type B/C follicles is possible. The lack of a strong inhibitory influence on type B/C follicles by larger follicles may explain, in part, why sections of bovine fetal ovaries incubated in vitro demonstrated an almost immediate increase in the number of primordial follicles transformed into primary follicles (Wandji et al., 1996).

A stimulatory influence from substances such as basic fibroblast growth factor must override the negative signals so that growth can continue (Parrott and Skinner, 1998). Therefore, growth could result from a two-step process. In step 1, follicles are converted from type B to type B/C; in step 2, the mixture of growth and inhibitory factors determine whether type B/C follicles continue to grow. A satisfactory explanation of how the individual type B or type B/C follicles are selected to respond remains unavailable. A possible intrinsic control mechanism is the ‘production line’ mechanism proposed by Henderson and Edwards (1968), who suggest that primordial follicles begin to grow in the same order in which they were formed. That is, the first oocytes entering meiotic arrest will be the first to be activated and the last oocytes entering meiotic arrest will not be activated until late in reproductive life. This hypothesis could be a partial explanation for the timing of activation and growth of individual follicles. However, it appears that although the production line may function to some extent, it may only do so for the first few weeks of life (Hirshfield, 1992). The only direct evidence in mature rats does not support the production line as being an important component of follicle activation throughout the majority of reproductive life (Meredith and Doolin, 1997).
Any changes in the ratio of type B:type B/C follicles that occur during reproductive life are unclear, although no differences were observed during the 150 day period in the present study. It is surprising that there was no significant decrease in type B follicles in the present study, although this may be because rats did not remain in the study long enough for a decrease to be observed statistically. However, it is possible that, at reproductive senescence, either type B/C or type B follicles are absent from the ovary. In addition, if one category but not the other is absent, this may have an adverse effect on fertility or embryonic mortality.

The relative numbers of type B to type B/C follicles reported in the present study (14 ± 3% type B follicles and 86 ± 3% of the population type B/C follicles) are in agreement with those reported by van Wezel and Rodgers (1996) in cattle, in which 82.5% of the population were type B/C follicles. The authors of the present study have conducted other experiments in which type B and type B/C follicles were evaluated from single sections. In one study, 4–5-month-old rats had 924 ± 67 type B and 1146 ± 116 type B/C follicles, and rats that were 9 months old had 790 ± 61 type B and 970 ± 86 type B/C follicles per ovary (S. Meredith, G. Dudenhoeffer and K. Jackson, unpublished). In this unpublished study, type B/C follicles still represented the major class of follicles, but made up only 55% of the population (unpublished), far less than the 86% reported in the present study. The primary cause for this discrepancy is that evaluation of single 7 µm thick sections of rat ovaries for type B/C follicles resulted in incorrect classification of them as type B follicles approximately 33% of the time (Meredith et al., 1999). The reverse error does not occur: that is, type B/C follicles are not misclassified as type B follicles. Therefore, counting by the traditional method results in a significant overestimate in the number of type B follicles and an underestimate of the number of type B/C follicles.

It could be argued that the results of the present study were obtained because BrdU-containing follicles were damaged and did not grow, since BrdU has been shown to be detrimental to DNA under certain conditions (Ackland et al., 1988; Raffel et al., 1989). In the present study, the mean number of labelled cells per follicle was slightly greater than one. There were usually two additional cuboidal cells that were not labelled in each follicle (that is, the number of cuboidal cells minus the number of labelled cuboidal cells). These unlabelled cuboidal granulosa cells should be able to undergo mitosis at the usual times because they would not be affected by BrdU uptake in adjacent cells. It also seems unlikely that BrdU had a major impact on the granulosa cells containing the label, because adverse long-term effects are usually due to mistakes in DNA replication (Morris, 1991). However, cuboidal cells in many of these follicles remained at the same stage throughout the experiment and apparently did not replicate.

In conclusion, the results of the present study demonstrate that DNA syntheses in rat granulosa cells is not a marker for initiation of growth and that follicles classified morphologically as type B/C consist of both growing and non-growing (arrested growth) follicles. Therefore, both B and B/C follicles in rats comprise the primordial pool of follicles in rats.

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