Fraction of proliferating cells in granulosa during terminal follicular development in high and low prolific sheep breeds

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Abstract — During terminal development of antral follicles, granulosa cells progressively lose their proliferative activity. Romanov (ovulation rate = 3) and Ile-de-France (ovulation rate = 1) breeds of sheep were compared for fractions of proliferating granulosa cells, determined by in vitro continuous $^{3}$H thymidine labelling. In both breeds, the fraction of proliferating cells decreased with increasing follicular size according to a sigmoid-shaped curve. After linearization, the slope of the regression line was higher (in absolute value) in Romanov, compared to Ile-de-France ewes ($p = 0.02$). In vivo FSH treatment led to a decrease in the slope of the regression line in Romanov ewes only ($p = 0.03$). These results suggest that during terminal follicular development (1) the rate of cell cycle exit is higher in granulosa cells of Romanov, compared to Ile-de-France follicles, and (2) Romanov granulosa cells are more responsive to exogenous FSH in term of proliferation. These mechanisms may underlie differential dynamics of follicular development in poly- and mono-ovulating breeds of sheep.

ovary / follicle / growth / cell cycle / ovulation rate

Résumé — Proportion de cellules de granulosa proliférantes dans les follicules ovariens en cours de développement terminal dans deux races ovines de proliférations différentes. Au cours du développement terminal des follicules ovariens, les cellules de granulosa perdent progressivement leur activité de prolifération. Les proportions de cellules de granulosa proliférantes ont été déterminées par une technique de marquage continue in vitro à la thymidine tritiée, dans deux races ovines de proliférations différentes, la race Romanov (nombre d’ovulations = 3) et la race Ile-de-France (nombre d’ovulations = 1). Dans ces deux races, les résultats ont montré que la proportion de cellules de granulosa proliférantes diminue selon une courbe sigmoïde quand la taille des follicules à antrum augmente. Après linéarisation, la pente de la droite de régression est plus forte (en valeur absolue) dans la race...
1. INTRODUCTION

During terminal follicular development, granulosa cells undergo terminal differentiation characterized by high expression and activity of luteinizing hormone (LH) receptors and steroidogenic enzymes, as well as drastic changes in peptid synthesis [10, 31, 32]. In sheep, the P450 aromatase enzyme is detectable in follicles larger than 2 mm diameter and aromatase activity increases dramatically when follicles enlarge to a preovulatory size of 6–7 mm diameter [12, 20, 38]. In a similar way, LH receptors are expressed by granulosa cells of follicles larger than 3 mm diameter, beyond which the sensitivity of granulosa cells to LH increases to high levels in preovulatory follicles [1, 3, 39, 40]. In parallel with these changes, granulosa cells progressively lose their proliferative activity [14]. In sheep, we have previously shown that the proliferative activity of granulosa cells decreases drastically in follicles between 2 and 6 mm diameter, and that this decrease corresponds to cell cycle exit [21, 24].

The mechanisms regulating the shift between proliferation and terminal differentiation in granulosa cells are largely unknown. First, the role of the follicle-stimulating hormone (FSH) in this transition has not been established. From available data, FSH both enhances the proliferative activity of granulosa cells and induces their terminal differentiation [31, 33, 37]. The mechanisms by which FSH can stimulate these opposite activities have not yet been understood. Second, the importance of this shift in the regulation of ovulation rate is not known. Clearly, the presence of the Fec gene of hyperprolificity in the Booroola ewe is associated with a more precocious maturation of follicles and a sharp decrease in the size of preovulatory follicles, which reaches only 3 mm diameter in FecFec ewes [9, 17, 18, 26]. Accordingly, follicles of prolific breeds ovulate at a smaller size associated with a lower number of granulosa cells, compared to mono-ovulating breeds of sheep, as it has been shown in the Belclare breed [30] and, to a lower extent, in Finnish Landrace [40], Finn [2, 6, 7] and Romanov [6] breeds. Investigations on the granulosa cell kinetics through a mathematical modelling approach [4] have suggested that the smaller number of granulosa cells in the preovulatory follicles of poly-ovulating breeds could be accounted for by a faster increase in the cell cycle exit rate. In addition, recent evidence indicate that the terminal maturation of follicles occurs earlier in the prolific Romanov (ovulation rate = 3) than in the non-prolific Ile-de-France (ovulation rate = 1) breed of sheep [1, 15]. Whether this earlier maturation is associated with an earlier loss of proliferative activity in Romanov granulosa cells has not been established.

The objectives of this study were (1) to determine the changes in the fraction of proliferating cells in granulosa cells during terminal follicular development, in the

ovaire / follicule / croissance / cycle cellulaire / taux d’ovulation
Proliferation of ovine granulosa cells were slaughtered without treatment. Nine Romanov and 9 Ile-de-France ewes (stimulated groups) were treated with intravaginal sponges impregnated with progestagen (fluorogestone acetate, 40 mg) for 14 days and primed with pFSH, administered intramuscularly 24 h (6 IU) and 12 h (5 IU) before slaughter at day 14. In a previous experiment, this treatment has been shown to increase proliferation rate in granulosa cells of Romanov follicles [23]. In each experimental group (n = 9 ewes), the granulosa cells isolated from 3 ewes were pooled for culture as described below. A total number of 12 independent cultures of granulosa cells (3 cultures per experimental group) were performed.

2. MATERIALS AND METHODS

2.1. Reagents and hormones

Intravaginal fluorogestone acetate sponges to synchronize estrus were obtained from Intervet (Angers, France). Porcine FSH from pituitary extract (pFSH activity = 1.15 × activity NIH pFSH-P1) used for injections in animals was obtained from Dr Y. Combarnous (Nouzilly, France). Granulosa cells were isolated and cultured in B2 medium [19] supplemented with bovine transferrin (10 mg L\(^{-1}\), Sigma Chemical Co., La Verpillière, France) and without cholesterol. Diethylstilbestrol (DES) and demecolcine (colcemid) were obtained from Sigma Chemical Co. Testosterone was purchased from Steraloids, Inc. (Wilton, NH). Ovine fetal serum was prepared from 130 days old fetuses, centrifuged, filtered through a 0.22 µm sterile filter and stored at –20 °C until use for culture. \(^{3}\)H]Thymidine (sp. act. 6.7 Ci mmol\(^{-1}\)) was purchased from Du Pont De Nemours (Les Ulis, France), and K5 emulsion for autoradiography was obtained from Ilford (St. Priest, France). Feulgen for staining of cells was purchased from Merck (Schuchardt, Germany).

2.2. Animals

Adult cyclic ewes (n = 36) were allocated to 4 experimental groups, according to breed and in vivo treatment. Nine Romanov and 9 Ile-de-France ewes (unstimulated groups) were slaughtered without treatment. Nine Romanov and 9 Ile-de-France ewes (stimulated groups) were treated with intravaginal sponges impregnated with progesterone (fluorogestone acetate, 40 mg) for 14 days and primed with pFSH, administered intramuscularly 24 h (6 IU) and 12 h (5 IU) before slaughter at day 14. In a previous experiment, this treatment has been shown to increase proliferation rate in granulosa cells of Romanov follicles [23]. In each experimental group (n = 9 ewes), the granulosa cells isolated from 3 ewes were pooled for culture as described below. A total number of 12 independent cultures of granulosa cells (3 cultures per experimental group) were performed.

2.3. Granulosa cell isolation and culture

For each culture, follicles from ovaries of 3 ewes of the same experimental group were dissected and allocated to 7 size classes, according to their diameter, i.e. class 1 [0.5–1.5 mm] (n = 17 to 40 follicles per culture), class 2 [1.5–2.5 mm] (n = 16 to 38 follicles per culture), class 3 [2.5–3.5 mm] (n = 5 to 14 follicles per culture), class 4 [3.5–4.5 mm] (n = 2 to 8 follicles per culture), class 5 [4.5–5.5 mm] (n = 2 to 3 follicles per culture), class 6 [5.5–6.5 mm] (n = 1 to 3 follicles per culture) and class 7 [6.5–7.5 mm] (n = 0 to 4 follicles per culture). The number of available follicles in each size class is subject to the variability between experimental groups. For each follicular class, granulosa cell isolation was performed as described previously [21]. The resulting suspensions of granulosa cells were seeded in quadruplicate at 10\(^5\) viable cells cm\(^{-2}\) in tissue culture chambers mounted on glass microslides (Lab-Tek, Nunc; Naperville, IL). In a previous study, we have shown that a seeding density varying between 0.5 and 2 × 10\(^5\) granulosa cells cm\(^{-2}\) does not affect the
estimation of the fraction of proliferating cells by $[^3\text{H}]$thymidine continuous labelling [24]. The viability of seeded cells was about 95%, as determined by trypan blue exclusion (0.125%, final concentration). Cultures were performed at 37 °C in an atmosphere of 5% CO$_2$, in B2 medium supplemented with ovine fetal serum (2%), testosterone (10$^{-7}$M) and DES (10$^{-7}$M), as described previously [21, 24]. After 24 h of culture, medium was changed before cells were submitted to continuous $[^3\text{H}]$ thymidine labelling for 48 h.

2.4. Continuous $[^3\text{H}]$thymidine labelling procedure

The procedure of Maekawa and Tsuchiya [13] was applied to determine the proportion of proliferating cells in granulosa cell cultures. This method, initially proposed to determine cell cycle parameters, is also appropriate for determining the percentage of dividing cells in cell populations. Its principle consists in the continuous administration of both colcemid to block cell replication, and $[^3\text{H}]$thymidine to label all proliferating cells. As a result, the percentage of labelled cells increases with time until a plateau is reached, after a delay corresponding to the duration of the G1 phase of the cycle. The height of the plateau is an estimation of the percentage of proliferating cells in the cell population [8]. For ovine granulosa cells, previous studies indicated that the plateau was reached after a 24 h to 48 h labelling period in our culture conditions [24]. Consequently, in $[^3\text{H}]$thymidine continuous labelling experiments, granulosa cells were incubated for 48 h in B2 medium supplemented as described above but without thymine, and in the presence of $[^3\text{H}]$thymidine (0.25 µCi·mL$^{-1}$) and colcemid (20 ng·mL$^{-1}$). At the concentration we used, treatment with colcemid does not impair cell viability and is efficient in blocking the proliferation of granulosa cells [24]. At the end of incubation, cells were washed three times with B2 medium (with thymine) and fixed with Bohm-Sprenger fixative (formaldehyde 15%, acetic acid 5%, methanol 80%) at 20 °C for 10 min. Slides were then stained with Feulgen and dipped in Ilford K5 emulsion diluted 1/1 (vol/vol) with distilled water, air-dried, and exposed for 6 days at 4 °C. The percentage of labelled cells (growth fraction) was estimated by counting the labelled and unlabelled cells in 40 randomly chosen microscopic fields (objective 40 ×) for each culture well, so that estimations were made on 1 000–2 000 cells per well. The precision of estimations, as assessed by the confidence interval, was higher than 0.97 for percentages between 20% and 80% and higher than 0.98 to 0.99 in the other cases.

2.5. Data analysis

For each of the four experimental groups, the growth fraction was expressed as a percentage of thymidine labelled cells. Data are presented as the means of the measurements performed with the different follicular classes in three independent cultures. The changes in the growth fraction with follicular size were fitted to sigmoidal curves with GraphPad PRISM Software (San Diego, CA, USA). After Logit transformation of the growth fraction, a linear regression was obtained in each of the four groups. The slopes of the regression lines were compared by ANOVA, followed by post-hoc comparisons with the Newman-Keuls test. This analysis was also used to compare intercepts with the ordinate axis ($x = 0$). Differences with $p > 0.05$ were not considered significant.

3. RESULTS

In each experimental group, the growth fraction of granulosa cells decreased from 70–85% in class 1 follicles to 1–4.5% in follicles of preovulatory size (class 6 in the
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The proliferation of ovine granulosa cells has been shown to be higher (in absolute value) in the group of Romanov unstimulated ewes, compared to the group of Ile-de-France unstimulated ewes ($p = 0.02$). FSH treatment led to a decrease in the slope of the regression line in Romanov ewes (Romanov stimulated vs. Romanov unstimulated, $p = 0.03$), but not in Ile-de-France ewes (Tab. I). There was no difference between experimental groups in the intercepts with the $y$ axis (Tab. I). To state the weight given to follicular classes with large

![Graphs](image)

**Figure 1.** Changes in the growth fraction of granulosa cells with increasing follicular diameter in follicles of Romanov and Ile-de-France ewes, with (stimulated groups) and without (unstimulated groups) in vivo FSH treatment.
dispersion of values between the three different cultures, analysis was also performed without including the follicular classes for which the standard deviation of the growth fraction was higher than the mean. Hence, new slopes of regression lines were calculated after omitting class 5 follicles in Romanov stimulated ewes, class 4 and class 6 follicles in Ile-de-France unstimulated ewes, and class 6 follicles in Ile-de-France stimulated ewes. These new slopes were not significantly different from those calculated including all data points (Romanov stimulated: $-0.872 \pm 0.095$, $n = 17$ vs. $-0.840 \pm 0.111$, $n = 20$, $p > 0.05$; Ile-de-France unstimulated: $-0.768 \pm 0.147$, $n = 21$, $p > 0.05$; Ile-de-France stimulated: $-0.990 \pm 0.108$, $n = 18$ vs. $-1.000 \pm 0.106$, $n = 20$). In agreement with the results obtained with all data points, the slope of the new regression line was higher (in absolute value) in the group of Romanov unstimulated ewes, compared to the group of Ile-de-France unstimulated ewes ($p = 0.04$). Furthermore, FSH treatment led to a decrease in the slope of the new regression line in Romanov ewes (Romanov stimulated vs. Romanov unstimulated, $p = 0.03$), but not in Ile-de-France ewes.

### 4. DISCUSSION

Whatever the breed and in vivo treatment, the growth fraction of granulosa cells decreased drastically during terminal follicular development. These results confirm previous data showing that the growth fraction is clearly lower in large follicles (5–7 mm diameter), compared to small follicles (1–3 mm diameter) in sheep [24]. They are also in agreement with results obtained in rat antral follicles, showing that the growth fraction decreases as follicular size increases [11]. Moreover, they support the existence of an inverse relationship between proliferation and differentiation in granulosa cells.

Unexpected high values of the growth fraction were observed in some large follicles (5 and 6 mm diameter follicles in Romanov stimulated and Ile-de-France unstimulated groups, respectively). Within the ovaries of each animal, the high heterogeneity of proliferative activity existing in granulosa cells between terminally developing follicles of similar size [25] can explain these results obtained on weak numbers of large follicles. This heterogeneity

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**Table I.** Relationship between the growth fraction (after Logit transformation) and the follicular diameter in Romanov and Ile-de-France follicles, with and without FSH treatment. Characteristics of the regression lines (slope and intercept with y axis) and values of the correlation coefficients ($r$) are indicated in the table. In each column, different letters mean significant differences between groups ($p < 0.05$). Exact values of $p$ are given in the text. S.E.M. are indicated in parentheses in the table.

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope</th>
<th>Intercept ($x = 0$)</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romanov unstimulated ($n = 17$)</td>
<td>$-1.345 \text{ a}$</td>
<td>$2.828 \text{ a}$</td>
<td>$-0.88$</td>
</tr>
<tr>
<td>Romanov stimulated ($n = 20$)</td>
<td>$-0.840 \text{ b}$</td>
<td>$2.159 \text{ a}$</td>
<td>$-0.87$</td>
</tr>
<tr>
<td>Ile-de-France unstimulated ($n = 21$)</td>
<td>$-0.768 \text{ b}$</td>
<td>$2.226 \text{ a}$</td>
<td>$-0.77$</td>
</tr>
<tr>
<td>Ile-de-France stimulated ($n = 20$)</td>
<td>$-1.000 \text{ ab}$</td>
<td>$2.596 \text{ a}$</td>
<td>$-0.91$</td>
</tr>
</tbody>
</table>
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likely results from differences in the developmental stage (rapid growth, plateau or regression) between follicles within a follicular wave of terminal development. Alternatively, the proliferative activity of granulosa cells in these large follicles may be variable between animals, in relationship with gonadotropin levels in blood just before the time of slaughtering. In bovine ovaries studied at the same day of the cycle, the percentage of mitosis in granulosa cells of antral follicles has been shown to vary more than threefold among animals [22].

Present data show differences in the proliferative activity of granulosa cells between both breeds. Indeed, the slope of the regression line linking the growth fraction of granulosa cells and the follicular diameter was higher in the prolific Romanov breed, compared to the non-prolific Ile-de-France breed. In contrast, no difference was observed between breeds after FSH treatment. These results suggest that in vivo (1) the rate of cell cycle exit during terminal follicular development is higher in Romanov, compared to Ile-de-France follicles; (2) Romanov granulosa cells are more responsive to exogenous FSH in term of proliferation. Previous results in Romanov and Ile-de-France breeds have indicated that, for a same follicular size, granulosa cells from Romanov have higher steroidogenic capacities and are more responsive to FSH and LH in vitro in terms of both cAMP and steroid production [1, 15]. Hence, we suggest that both the loss of cell proliferation and terminal differentiation would occur earlier in granulosa cells of Romanov, compared to Ile-de-France, follicles. In the prolific Booroola Fec+ and FecFec ewes, increased ovulation rate is clearly associated with precocious maturation of a large number of small antral follicles which reach the preovulatory stage at a smaller size than ++ follicles [9, 17]. Accordingly, the prolific Inverdale I+ ewes are characterized by a higher responsiveness of granulosa cells to LH in term of cAMP production, occurring at smaller follicular diameters compared to non-prolific ++ ewes [36]. This earlier terminal differentiation is accompanied by an earlier loss of granulosa cell proliferation in Fec carrier antral follicles [5], and a lower number of granulosa cells per preovulatory follicle in Booroola Fec carrier [16] and in Inverdale I+ ewes [36], compared to non-prolific ++ ewes. Together with our data, these results suggest that high ovulation rate in prolific breeds is associated with a more precocious maturation of granulosa cells. The mechanisms underlying this relationship remain to be investigated.

In Romanov ewes, FSH treatment both increased the size of the largest follicles from 6 to 7 mm diameter, and decreased the slope of the regression line linking the growth fraction of granulosa cells and follicular diameter. These results suggest that FSH has enhanced the growth of large antral follicles in this breed. This stimulating effect of FSH could be mediated, at least partly, by an increase in the growth fraction of granulosa cells in follicles of 4–5 mm diameter, as it can be seen in Figure 1. In contrast, in small follicles of 1–2 mm diameter, no effect of FSH treatment on the growth fraction of granulosa cells was observed. This difference in the responsiveness of granulosa cells from large and small antral follicles to FSH may be due to a difference in the dynamics of the growth fraction after FSH administration, or/and to a difference in the mechanisms of action of FSH. Recent data indicate that FSH induces in a rapid and direct way an increase in the expression of cyclin D2 in hypophysectomized rats as well as in rat granulosa cells cultured in serum-free conditions [33]. Interestingly, ovarian follicles of cyclin D2-/- mice do not undergo rapid growth in response to FSH, but do express markers of cell cycle exit and terminal differentiation [33, 37]. Collectively, these results suggest that FSH would exert two types of effects on granulosa cell proliferation. The first effect of FSH is rapid, direct and cyclin D2 dependent, and would induce a shortening of the duration of the cell cycle by stimulating the transition G1–S
in granulosa cells, as previously suggested [27–29]. In Romanov ewes, incubation of granulosa cells with FSH for 2 h increases thymidine incorporation and this rapid effect is observed in cells from small as well as large antral follicles [20]. The second effect of FSH is delayed, likely indirect and cyclin D2 independent, and would induce a re-entry in the cell cycle or slow down cell cycle exit by acting on the G0–G1 transition in granulosa cells. Particularly, such an effect of FSH might be mediated by growth factors originating from granulosa or/and theca cells, as suggested by data obtained with hamster follicles [34, 35]. Accordingly, in follicles of Romanov ewes, we propose that the stimulating effect of a 24 h FSH treatment on growth fraction would be indirect, likely mediated by changes in growth factor synthesis or bioavailability in follicular cells, and that the kinetics of response of granulosa cells would be modulated by their stage of differentiation.

In conclusion, the results of this study have shown effects of breed, FSH treatment and maturation stage of follicles on the fraction of proliferating cells in granulosa. The prolific Romanov breed was characterized by an earlier decrease in growth fraction during follicular growth and a higher sensitivity to FSH in vivo. Further investigations are needed now to determine the mechanism by which FSH exerts its effects on granulosa cell proliferation. Particularly, it remains to determine whether FSH is able to slow down the rate of cell cycle exit or stimulate the re-entry of granulosa cells in the cell cycle.

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REFERENCES


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