Growth kinetics of the granulosa cell population in ovarian follicles: an approach by mathematical modelling

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Abstract. This paper describes, from a mathematical viewpoint, the cellular changes in the granulosa of ovarian follicles during their terminal development. A dynamic model takes into account the processes of (1) cell division, (2) exit from the cell cycle towards differentiation, and (3) apoptotic cell death. Proliferative cells leave the cycle in an irreversible way. The risk of entering apoptosis applies to non-cycling cells. Changes in the cell numbers and in the growth fraction are derived from differential equations. The transitions between the different cell states are ruled by time-dependent rates. Numerical applications of the model concern ovulating and degenerating ovarian follicles in the ewe. The main feature of the ovulating case is the progressive exhaustion of the proliferating compartment for the benefit of the non-cycling cells. From an initial mainly proliferative state the granulosa progressively switches to a highly differentiated state, so that the growth fraction continuously decreases. In the atretic cases, the pattern of changes in the total viable cell number is influenced by the follicular age at the onset of the apoptotic process and by the intensity of the cell death rate. As apoptosis affects the non-cycling cells, the growth fraction is no longer strictly decreasing. The sensitivity of the model to the parameters is studied in a more general framework than the granulosa cell population.

In mammals, the pool of oocytes available for a female through her reproductive life is constituted very early in life. Each oocyte of this pool is surrounded by only one layer of somatic cells, the whole making up a primordial follicle (Mauléon 1969). Follicles continuously leave this pool to enter a process of growth and functional maturation, called folliculogenesis, until the pool is fully depleted. The biological meaning of folliculogenesis is to free fertilizable oocyte(s) at the time of ovulation.

The initiation of growth in primordial follicles is characterized by an increase in the oocyte size concomitant with the surrounding somatic cells proliferating to build an avascular tissue called granulosa (Hirshfield 1991). Growth is then closely related to the proliferation of granulosa cells. In follicles with five to six layers of granulosa cells, perifollicular cells of the ovarian stroma differentiate to form a conjunctive, vascularized tissue called theca.

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Simultaneously, the granulosa becomes hollow with a cavity containing follicular fluid, called the antrum, in which secretions of follicular cells, as well as serum factors, accumulate. The mitotic index of the granulosa cells goes on increasing with follicular diameter before reaching a peak in small antral follicles.

During the terminal part of follicular development, vascularization develops, while granulosa cells progressively lose their ability to proliferate and acquire a high steroidogenic activity (Mariana *et al.* 1991). The mitotic index decreases until mitotic figures can be scarcely detected in pre-ovulatory follicles, so that growth is no longer due to cell proliferation but to the tremendous enlargement of the antrum (Figure 1).

The total duration of follicular growth largely exceeds the duration of a menstrual or oestrous cycle. While growth of pre-antral follicles is very slow (several months), terminal development is achieved in a few days. Once initiated, follicular growth is a continuous process without resting phases, ending at ovulation. Yet, very few follicles will reach ovulatory size; most of them will undergo a degeneration process, called atresia, due to granulosa cells entering apoptosis when their trophic requirements are not met (Hsueh, Billig & Tsafiriri 1994). The control of granulosa cell cycles by the pituitary gonadotrophins, FSH (follicle-stimulating hormone) and LH (luteinizing hormone), is a critical point in the balance between proliferation, differentiation and apoptosis, hence in the regulation of terminal

Figure 1. Antral growth in ovarian follicles. Follicles are spheroids made up by, from outside inwards, (i) the conjunctive and vascularized theca (light grey), (ii) the avascular granulosa (dark grey) and (iii) the liquid antrum. The oocyte is surrounded by granulosa cells forming a protuberance in the antrum. During the terminal growth of ovarian follicles, the thickness of the granulosa remains almost constant, while antral diameter increases dramatically and theca vascularization develops to supply nutrients to follicular cells. The growth of antrum can be monitored through ultrasonographic device.

follicular development. Gonadotrophin secretion is, in turn, modulated by granulosa cell products such as oestradiol and inhibin.

A few mathematical models have already described the ovarian follicle using the morphological stage it belongs to (Faddy, Jones & Edwards 1976), its age (Read, Mariana & de Reviers 1972) or its maturity level (Lacker & Akin 1988). None has been concerned with the characterization of the ovarian follicle in terms of granulosa cell kinetics, although the fate of a follicle is mainly determined by the cellular changes in its granulosa.

This paper describes, from a mathematical viewpoint, the cellular changes in the granulosa of ovarian follicles during their terminal development. We built a dynamic model, which takes into account the processes of (1) cell division, (2) exit from the cell cycle towards differentiation, and (3) apoptotic cell death. The respective numbers of proliferative, differentiated and apoptotic cells are ruled by ordinary differential equations. The cell cycle exit rate and the rate of entering apoptosis are time-dependent, that is, they vary with follicular age. Assumptions underlying the flows of cells are that proliferative cells are progressively driven to leave the cell cycle under cumulative exposure to FSH, while non-cycling cells are exposed to the risk of entering apoptosis if the supply of FSH becomes lower than that which the follicle needs. In the model, these assumptions amount to increasing the cell cycle exit rate with follicular age and to triggering apoptosis when the follicular age passes a threshold. From the model equations, it is also possible to deduce the number of viable cells and the growth fraction.

We designed a numerical application of the model in the ovine species. We were interested in ewe follicles just when the proliferation peak in the granulosa ends, which determines the initial conditions for the equations and corresponds to a 1 mm follicular diameter. We adjusted the values of the exit rate parameters on the changes in the number of viable cells during follicular development from the proliferation peak through ovulation. We used the fitted values to perform computer simulations for ovulating as well as for atretic follicles. Beyond the specific case of granulosa cells, the study of the model highlights compatibility relations between the parameter values.

DESCRIPTION OF THE MODEL

The granulosa cell population consists of proliferating, differentiated and apoptotic cells (Figure 2). Proliferative cells leave the division cycle in an irreversible way, according to the antagonism between proliferation and differentiation observed during terminal follicular development. The apoptosis risk applies to non-cycling cells. This assumption is substantiated by flow cytometry studies on granulosa cells showing the appearance of apoptotic cells at the expense of cells in the G_0/G_1 phase (Blondin, Dufour & Sirard 1996). Entry into apoptosis

Figure 2. Flow chart of the model. Rectangles represent compartments, straight-lined arrows represent cell flows between compartments, the circle arrow marks cell division. Letters correspond to the instantaneous flow rates. μ , rate of cell division; $\delta(t)$, rate of cell cycle exit; $\alpha(t)$, rate of cells entering apoptotic death; and $w(t, s)$, rate of phagocytose for a cell that entered apoptosis s h ago.

is also considered to be irreversible. Apoptotic cells gradually disappear from the follicle, as it is well established that apoptotic cells are quickly phagocytized by macrophages or neighbouring cells (Hsueh *et al.* 1994). The kinetics of the granulosa cell population is expressed as a function of follicular age by the following system of differential equations:

$$\frac{dN_p(t)}{dt} = (\mu - \delta(t)) N_p(t) \quad (1)$$

$$\frac{dN_d(t)}{dt} = \delta(t) N_p(t) - \alpha(t) N_d(t) \quad (2)$$

$$\frac{dN_a(t)}{dt} = \alpha(t) N_d(t) - \int_0^s w(\tau) \alpha(t-\tau) N_d(t-\tau) d\tau \quad (3)$$

where $N_p(t)$ = number of proliferating cells, $N_d(t)$ = number of differentiated cells, $N_a(t)$ = number of apoptotic cells, μ = rate of cell division ($\ln 2/T_c$), $\delta(t)$ = rate of leaving the cell cycle, $\alpha(t)$ = rate of entering apoptotic death, $w(t)$ = probability density function for the delay before phagocytosis, and s = maximal delay before phagocytosis.

The rate of cell division μ is inversely proportional to the duration of the cell cycle T_c , which was assumed to remain constant throughout follicular development. Indeed, in the ewe, *in vitro* experiments with continuous thymidine labelling have shown that the delay before reaching a plateau in the proportion of unlabelled cells is similar between small and large antral follicles (Monniaux, Pisselet & Fontaine 1994). As this delay can be assimilated to the duration of the G_1 phase (Maekawa & Tsuchiya 1968), the most variable phase in the cycle, we kept the average cycle duration identical between follicles. We also neglected the variability between cells. The exit rate from the cycle $\delta(t)$ was assumed to increase with follicular age. Choosing a constant rate would have led to an exponential growth pattern for the proliferative compartment. Instead, an increasing function allows a two-phased pattern beginning with an increase phase, as long as the exit rate is lower than the net growth rate, and ending with a decrease phase. On a physiological ground, the numerical values taken by the exit rate are necessarily limited. The lower bound corresponds to the maximal rate of cells remaining in the cycle and cannot be negative, while the upper bound is limited by the duration of the G_1 exit window relative to the whole cycle duration. As we presumed an S-shaped curve, with asymptotic bounds, we used a logistic, possibly asymmetric, function:

$$\delta(t, \theta) = \delta_2 + \frac{\delta_1 - \delta_2}{(1 + \exp(\delta_3 + \delta_4 t))^{\delta_5}} \quad (4)$$

where $\theta = (\delta_1, \delta_2, \delta_3, \delta_4, \delta_5)$ vector of real parameters.

In the model, cell entry into apoptosis starts after the follicular age has reached a threshold (t_A). Before this time, the rate of entering apoptosis $\alpha(t)$ is null. Afterwards, the rate increases with follicular ageing in a quadratic way. This formulation allows for the acceleration of the apoptotic process with ageing:

$$\alpha(t) = \begin{cases} 0 & \text{if } t \leq t_A \\ a(t - t_A)^2 & \text{if } t > t_A \end{cases} \quad (5)$$

The integral term in equation (3) ensures that apoptotic cells do not accumulate inside the follicle. The pragmatic formulation of the probability density function for the phagocytosis times $w(t)$ respects the idea that the probability for an apoptotic cell to be phagocytosed increases from its entering apoptosis until a maximal delay:

$$w(t) = \frac{n}{s^n} t^{n-1} \quad (6)$$

where n = integer parameter, and s = maximal delay before phagocytosis.

From equations (1) and (2), one can deduce the kinetics of the viable (non-apoptotic) granulosa cells, as well as the growth fraction, representing the relative number of proliferating cells in the viable cell population:

$$\frac{dN_T(t)}{dt} = \mu N_p(t) - \alpha(t) N_d(t) \quad (7)$$

$$GF(t) = \frac{N_p(t)}{N_T(t)} \quad (8)$$

$$\frac{dN_T(t)}{dt} = N_T(t) \{ GF(t) [\mu + \alpha(t)] - \alpha(t) \} \quad (9)$$

where $N_T(t)$ = viable cells, and $GF(t)$ = growth fraction.

If the apoptotic process can be neglected, the growth fraction can be deduced from the number of viable cells in a simple manner:

$$GF(t) = \frac{dN_T(t)/dt}{\mu N_T(t)} \quad (10)$$

The cell kinetics parameter μ only acts as a scale parameter on the growth fraction. As $GF(t)$ must not exceed unity, there is a minimal acceptable value for μ , hence a maximal value for T_c , compatible with a given series $N_T(t)$.

APPLICATION TO THE CELLULAR CHANGES IN THE GRANULOSA OF EWE FOLLICLES

Cellular changes in the granulosa of ovulating follicles

Granulosa cell numbers

As most of the information concerning follicular development, granulosa cell counts are available as a function of follicular diameter. We used the cell-diameter relation given by Tsonis, Carson & Findlay (1984b), who assumed that the healthiest, potentially ovulating, follicles have the highest number of cells for a given follicle diameter. The experimental regression formula has a logarithmic expression:

$$N_T(D) = 206612 + 3229327 \ln D \quad D \geq 1 \quad (11)$$

where $N_T(D)$ = total granulosa cell number, and D = diameter of follicles after dissection (mm).

In order to obtain the cell counts as a function of time, we need to know the relationship between the age of the follicle and its diameter. This average relation is not directly accessible during the whole period from the proliferation peak in the granulosa until ovulation, but can be established by coupling piecemeal information:

(1) *In vivo* cauterization experiments of ovarian follicles have shown that the ovulatory follicle(s) is (are) selected from the follicles reaching a 2 mm diameter at luteolysis (Tsonis *et al.* 1984a).

(2) Ultrasonographic studies have enabled the day-to-day monitoring of antral follicles from an inner diameter exceeding 2 mm size until the ovulatory size of 7 mm in western white faced (merino-cross) ewes (Ravindra *et al.* 1994). The growth rate has been estimated at 1.2 mm per day. Once the diameter has reached its maximal size, it remains at a plateau until ovulation (or possibly late atresia).

(3) Cell kinetic studies have shown that the duration of follicular growth from the end of the mitotic index peak in the granulosa until ovulation is about 200 h in mono-ovulating merino ewes (Turnbull *et al.* 1978).

Putting these facts together, we propose an average regression formula for the radial growth in ewes of merino-related breeds:

$$D(t) = 0.90 + 7.10 \{1 + \exp(6.41 - 0.04t)\}^{-0.66} \quad (12)$$

where D = diameter of follicles after dissection (mm), and t = time (h).

The time origin for the follicular age is made coincident with the proliferation peak and corresponds to a 1 mm diameter. The relation also derives from the fact that the delay between 1 mm and 7.5 mm diameter (197 h), provided by the cell kinetics studies, is twice the delay between 2.5 mm diameter and 7.5 mm diameter, provided by the ultrasonographic studies. The thickness of the cellular corona remaining around the antrum after dissection can be considered constant, so that the diameter after dissection runs from 2.5 mm to 7.5 mm while the inner diameter runs from 2 mm to 7 mm. Besides, the maximal diameter always remains beneath 8 mm. These constraints are sufficient to determine the numerical values of the five parameters in relation (12).

Numerical calibration of the model

In the case of ovulatory follicles, the trophic requirements of granulosa cells are fulfilled throughout follicular development. Even if a few apoptotic cells may be detected in healthy follicles, the apoptotic process can be neglected so that the $\alpha(t)$ rate in equation (2) remains null and the expression of the total granulosa cell number when combining equations (1) and (7) simplifies to:

$$\frac{dN_T(t)}{dt} = \mu N_p(0) \exp \int_0^t (\mu - \delta(\tau, \theta)) d\tau \quad (13)$$

The growth rate μ and the initial values $N_T(0)$ and $N_p(0)$ were held fixed. We considered the initial growth fraction $N_p(0)/N_T(0)$ to equal 0.75. We have indeed recently measured this value after continuous thymidine labelling of cultured granulosa cells from 1 mm diameter follicles (Monniaux *et al.* 1997). The corresponding cycle duration T_c was 11.54 h ($\mu = 0.06 \text{ h}^{-1}$). The values of the θ parameter vector were estimated from a series of granulosa cell numbers. Cell numbers as a function of follicular age were obtained by combining the cell-diameter relation with the diameter-age relation (see previous section). We mimicked the measurement error by adding a Gaussian centred noise of variance 10^5

cells, in order to rebuild observed values. The regression model describing these data is written as follows:

$$Y_i = N_T(t_i) + \varepsilon_i \quad i = 1, \dots, n \quad (14)$$

where Y_i is the granulosa cell number observed at time t_i , $N_T(t_i)$ is solution of equation (13) and the ε_i s are Gaussian errors with mean zero and constant variance. n (50) is the size of the series.

We used nls2, a non-linear regression program implemented as an extension of the statistical system S-PLUS (Bouvier & Huet 1994), to estimate the model parameters by minimizing the residual sum of squares:

$$S = \sum_{i=1}^n (N_T(t_i) - Y_i)^2 \quad (15)$$

We evaluated the equations required to define the model in nls2 through a C program calling to the cfNAG routine d01akf (details in the Appendix).

The values of both fixed and estimated parameters were used to perform numerical simulations of the changes in the proliferating $N_p(t)$ and differentiated $N_d(t)$ cell numbers. The differential equations (1) and (2) were integrated by means of a Runge-Kutta method of order 4, with a step of 0.1 h, which we also implemented in C language.

Results of the estimation and simulation procedures

The estimated values of the parameters are presented in the first row of Table 1 and the corresponding curve for the exit rate is shown in Figure 3. The lower asymptote δ_2 roughly equals half the value of μ , while the upper asymptote δ_1 roughly reaches twice the value of μ . The speed of increase in $\delta(t)$ is maximum at the inflection point I, whose coordinates are $t = 156$ h and $\delta(t) = 0.085$ h⁻¹.

The data points are plotted on Figure 4 together with the adjusted curve for the total cell number and its decomposition into proliferative and differentiated cells. After an initial exponential increase phase, the number of proliferative cells decreases for the benefit of the non-cycling population. The number of differentiated cells only stops increasing significantly when too few proliferative cells remain in the granulosa. The pattern of the growth fraction is shown in the insert of Figure 4. Starting from the initial value of 0.75, the growth fraction continuously decreases until it becomes quasi null when almost all proliferating cells have vanished. The relation between the total number of granulosa cells and the composition of the granulosa population is illustrated in Figure 5.

Table 1. Division rate μ and cycle exit rate $\delta(t, \theta)$

$GF(0)^a$	μ^b	Exit rate parameters				
		δ_1^b	δ_2^b	δ_3^c	δ_4^c	δ_5^{cd}
0.75	0.06	0.124	0.031	6.28	-0.036	0.5
1.0	0.045	0.109	0.016	6.28	-0.036	0.5
0.5	0.09	0.154	0.061	6.28	-0.036	0.5

^aInitial growth fraction.

^bIn h⁻¹.

^cDimensionless parameter.

^dNon-estimated value.

Changing the values of the model parameters can have great repercussions on the patterns of both the viable cell number and the growth fraction:

(1) In the framework of our application, the maximal value of the exit rate (roughly δ_i) exceeds the net growth rate (μ). Otherwise, the total number of cells would increase indefinitely, in an exponential way, and would never reach a plateau. In a general manner, the number of cells at the end of the simulation is influenced by the time at which the exit rate exceeds the division rate (see the point E where $\delta(t) = \mu$ in Figure 3). As soon as the proliferative capital is broken into, the maximal number of cells attainable is determined.

(2) In the case of ovulating follicles, the growth fraction is a monotonic decreasing function of time, whose derivative always remains negative. This is specifically true at the time origin, when the condition on the sign of the growth fraction derivative can be expressed in the form of an order relation between the cycle duration T_c and the initial values of the growth fraction $GF(0)$ and of the exit rate $\delta(0)$:

$$\left(\frac{dGF(t)}{dt} \right)_{t=0} < 0 \Leftrightarrow \delta(0) > \frac{\ln 2}{T_c} (1 - GF(0)) \quad (16)$$

Should the relation (16) be not respected, the growth fraction would initially either stay at a plateau (in case of equality), or even increase (in case of the opposite inequality). Thus this

Figure 3. Changes in the cell cycle exit rate $\delta(t)$ as a function of the follicular age t compared to the constant value of the cell division rate μ (horizontal line). E, intersection point where $\delta(t) = \mu$; I, inflection point.

relation is helpful to assign initial values to the parameters, in such a way as to make them consistent with one another and with the available information on the growth fraction.

(3) Different combinations of the parameters δ_1 , δ_2 , μ and $GF(0)$ are compatible with a given series of cell numbers. Altering the values of $GF(0)$ or μ does not imply that one starts a whole estimation procedure again, but just leads one to adapt the scale of $\delta(t)$ without affecting its shape. Leaving unchanged the values of the parameters ruling the abscissa of the inflection points (δ_3 , δ_4 , δ_5), three relations are kept constant: (i) the product $\mu GF(0)$ of the division rate by the initial growth fraction; (ii) the difference $\delta_1 - \mu$ between the upper asymptote and the division rate; (iii) the difference $\delta_1 - \delta_2$ between the two asymptotes. Figure 6 illustrates the scaling of $\delta(t)$ for two values of $GF(0)$ leaning from one side of the previously chosen value ($GF(0) = 0.75$) to the other. The different sets of parameters are gathered in Table 1. The possibility of scaling $\delta(t)$ provides rapidly the different values of the parameters corresponding to a range of values for the initial growth fraction or the cell cycle duration, in case they are not known with precision.

Cellular changes in the granulosa of atretic follicles

We simulated the courses of atretic follicles by taking back the numerical values estimated in the ovulatory case for the exit rate $\delta(t)$ and adding the occurrence of apoptosis. We studied different follicular ages for the onset of apoptosis (t_A): 50 h, 100 h and 150 h. In each case, we

Figure 4. Main plot: changes in the number of viable cells $N_T(t)$ (solid line), proliferating cells $N_p(t)$ (dotted line) and differentiating cells $N_d(t)$ (dashed line), as a function of the follicular age t . Insert: changes in the growth fraction $GF(t)$ of the granulosa cell population as a function of the follicular age t .

tested two different intensities of the quadratic coefficient a in equation (5), corresponding to the apoptosis rate reaching 0.05 h^{-1} or 0.08 h^{-1} within 36 h. The maximal delay before phagocytosis (s) was empirically set at 12 h.

As previously, we performed the numerical simulations of the model through a computer program written in C language. The integration of the differential equations (1)–(3) uses a Runge–Kutta method of order 4, with a step of 0.1 h. The integral expression ruling the disparition of apoptotic cells in equation (3) is solved by calling to the subroutine d01gaf of the cfNAG library. Figures 7 and 8 compare different courses of atretic follicles with the course of the ovulating follicle. The pattern of the changes in the total cell number is influenced by the follicular age at the beginning of the apoptotic process. The intensity of the cell death rate also affects the speed at which the follicle degenerates (Figure 7). If there are still proliferative cells in the granulosa at the onset of apoptosis, cell depletion first slows down the rate of increase in the total number of viable cells. By the time the number of non-cycling cells committed to apoptosis overcomes the number of dividing cells ($\alpha(t) N_d(t) > \mu N_p(t)$ in equation (7)), the rate of increase becomes negative and the total cell population is reduced. Consequently, when apoptosis occurs before the exhaustion of the

Figure 5. Changes in the cellular composition of the granulosa cell population of the ovulating follicle. The sizes of the pie-charts are proportional to the number of viable cells in the granulosa. The horizontally-striped portion represents the number of proliferating cells $N_p(t)$, while the obliquely striped portion represents the number of differentiating cells $N_d(t)$. t is the follicular age.

Figure 6. Repercussions of altering the initial growth fraction $GF(0)$. Upper plot: changes in the growth fractions as a function of the follicular age t . Lower plot: changes in the cell cycle exit rates as a function of the follicular age t compared to the constant values of the cell division rates (horizontal lines). Long-dashed curves, $GF(0) = 1$; short-dashed curves, $GF(0) = 0.5$; solid lines, $GF(0) = 0.75$. Each curve corresponds to the same series of viable cell numbers shown in Figure 4.

proliferative compartment, cell depletion is smoother than when it occurs on a fully non-cycling population. The onset of apoptosis also modifies the pattern of changes in the growth fraction, which is no longer strictly decreasing (Figure 8).

DISCUSSION

We approached the modelling of follicular development from the granulosa cell kinetics viewpoint, because the regulation of the cellular changes in the granulosa is a key issue. As far as we know, this is the first attempt to build a mathematical model of follicular development on a cellular basis. Previous models characterized follicles by their morphological stage (Faddy, Jones & Edwards 1976) or by their age (Read, Mariana & de Reviers 1979). Their main purpose was to describe the stage or age-structure of the follicular population in the ovaries, given that the atresia risk for any follicle is ruled by statistical laws. Such models were concerned with follicles from the time they leave the pool of primordial follicles until ovulation or atresia. We instead focused on a much shorter period of follicular development, starting at the end of the proliferation peak in the granulosa. Lacker & Akin (1988) was also interested in the terminal part of follicular maturation. They characterized ovarian follicles by a unique maturity variable whose changes are ruled by a deterministic, yet empirical,

Figure 7. Comparative changes in the viable cell numbers of the ovulating follicle (solid line) and of atretic follicles (dashed lines), as a function of the follicular age t . Cell numbers are expressed in millions. The different atretic trajectories differ in the time of apoptosis onset and the speed of increase in the rate of cells entering apoptosis. The different times of apoptosis onset were: 50 h (left pair of curves), 100 h (middle pair of curves) and 150 h (right pair of curves). Long-dashed curves and short-dashed curves correspond to a rate reaching respectively 0.05 h^{-1} or 0.08 h^{-1} within 36 h.

function allowing for the emergence of a selection process between follicles. We emphasized the individual characterization of the follicle by means of a multidimensional cellular description. We gave a functional, even mechanistic basis to the formulation of the rates of change in each descriptive component, that is in each category of granulosa cells. Our dynamic model rules the flows of cells from one cellular state to another by integrating basic knowledge on the cell cycle physiology, without taking care in detail of the biochemical pathways leading the cell to proliferation, differentiation and apoptosis.

The model is able to retrace the route of the ovulatory follicle during the terminal part of its development as well as deviation from this route corresponding to follicle degeneration. From an initial mainly proliferative state, the granulosa of the pre-ovulatory follicle progressively switches to a highly differentiated state, so that the growth fraction continuously decreases. The follicle awaits ovulation with an almost constant number of cells. In this sense, it has reached a cellular steady state. However, this is a temporary state, because the onset of ovulation will completely remodel the follicle into a corpus luteum. The number of cells that is reached at the steady differentiated state is directly related to the capacity of oestradiol secretion, through which the follicle takes part in the endocrine interactions between the ovaries and the pituitary gland. As far as atretic follicles are concerned, there is no stable steady state reached at the time when the number of cells committed to apoptosis becomes

Figure 8. Comparative changes in the growth fraction of the ovulating follicle (solid line) and of atretic follicles (dashed lines), as a function of the follicular age t . The different atretic trajectories differ in the time of apoptosis onset and the speed of increase in the rate of cells entering apoptosis. The different times of apoptosis onset were: 50 h (left pair of curves), 100 h (middle pair of curves) and 150 h (right pair of curves). Long-dashed curves and short-dashed curves correspond to a rate reaching respectively 0.05 h^{-1} or 0.08 h^{-1} within 36 h.

equal to the number of dividing cells. Nevertheless, this moment is a critical point in the follicular route towards the fully degenerated state.

We performed a numerical calibration of the model for an average ovulating follicle, in sheep. We chose to study the cellular changes as a function of the follicular age rather than of its diameter. Indeed, the diameter is only an observation variable, not an explicative one; it is the diameter growth that proceeds from the cellular changes, rather than the contrary. Secretions emanating from the differentiated cells, adding to plasmatic transudation, are responsible for the swelling of the antrum, hence for the increase in the inner follicular radius, while cell proliferation maintains an almost constant cell layer thickness around the antrum. The accuracy of the calibration would be improved if we could acquire more information about the temporal pattern of follicular growth. However, the individual characterization of the cellular changes in relation to the ageing of the follicle is beyond reach, as it is impossible to obtain simultaneously information about the cell number and about the growth kinetics of a given follicle: the counting of granulosa cells is an invasive measure, which prevents the pursuit of normal follicular development.

The numerical calibration was designed on data collected in ewes of merino or merino-related breed. It could apply to other breeds or species in which the cycle duration of granulosa cells, cell counts and follicular radius growth kinetics data are available. In poly-ovulating ewes, both the diameter and the total cell number of ovulating follicles are often smaller than they are in mono-ovulating ewes (McNatty *et al.* 1986). The framework of the model enables one to explain this feature either (1) as differences in the cell cycle duration or (2) as differences in the sensitivity of granulosa cells to FSH-induced differentiation. In both cases, the cell cycle exit rate would exceed the division rate earlier in the poly-ovulating ewes. In the former case, the division rate would be lower and hence the cycle duration of granulosa cells longer. The latter case can be achieved in several ways. The relative length of the cycle exit window could be longer. Alternatively, the exit rate could reach its maximal slope earlier. In order to test these predictions, we are implementing new experiments. They consist of measuring the growth fraction in different breeds of sheep, in basal as well as in FSH-stimulated conditions. They will confirm whether the decrease in the growth fraction is sharper in poly-ovulating ewes and will give information about FSH-induced differentiation in different breeds. In any event, these intra-species differences correspond to different strategies of the follicle exploiting its proliferative capital, which directly influences its final number of fully differentiated, oestradiol-producing cells.

The amount of plasmatic oestradiol aggregates the contributions of every secreting ovarian follicle to regulate the gonadotrophin secretion by the pituitary gland, so that the composition of the follicular population determines *in fine* the circulating levels of gonadotrophins. The dynamic equilibrium between proliferation, differentiation and apoptosis in the granulosa of one given follicle is controlled by the interactions between the gonadotrophic environment and its own maturity level. Consequently, the trajectories of the follicles are not independent: the trajectories of atretic follicles are driven by those of the pre-ovulatory ones. The selection process ruling the ovulation rate arises from this mutual dependence. Hence, the next step in modelling will be to couple the changes in the granulosa cell populations of the different follicles.

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APPENDIX

Estimation procedure using nls2

nls2 is a set of SPLUS functions and programs (available by <http://www-bia.inra.fr/> or by ftp www-bia.inra.fr/pub/log/nls2) useful for estimating the parameters of a non-linear regression model over a given set of observations. The regression function can be defined as the solution of a system of differential equations depending on unknown parameters. The stages of the estimation procedure with nls2 are summarized in the following diagram:

The SPLUS function calling to nls2 requires arguments such as the set of observations, the name of the C file describing the model and information about initial conditions. The C file computes the derivative $dN_i(t)/dt$ (see equation (13)) and its own partial derivatives in

terms of each parameter of the exit rate $\delta(t)$: $\partial/\partial\theta (dN_T(t)/dt)$. It calls to the NAG routine d01akf to integrate every derivatives. The outputs are provided to the optimization procedure of nls2, which updates in turn the current values of the parameters until a stopping criterion is satisfied.