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Review

Multi-scale modelling of ovarian follicular development: From follicular morphogenesis to selection for ovulation

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In this review, we present multi-scale mathematical models of ovarian follicular development that are based on the embedding of physiological mechanisms into the cell scale. During basal follicular development, follicular growth operates through an increase in the oocyte size concomitant with the proliferation of its surrounding granulosa cells. We have developed a spatio-temporal model of follicular morphogenesis explaining how the interactions between the oocyte and granulosa cells need to be properly balanced to shape the follicle. During terminal follicular development, the ovulatory follicle is selected amongst a cohort of simultaneously growing follicles. To address this process of follicle selection, we have developed a model giving a continuous and deterministic description of follicle development, adapted to high numbers of cells and based on the dynamical and hormonally regulated repartition of granulosa cells into different cell states, namely proliferation, differentiation and apoptosis. This model takes into account the hormonal feedback loop involving the growing ovarian follicles and the pituitary gland, and enables the exploration of mechanisms regulating the number of ovulations at each ovarian cycle. Both models are useful for addressing ovarian physio-pathological situations. Moreover, they can be proposed as generic modelling environments to study various developmental processes and cell interaction mechanisms.

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Introduction

In mammals, the ovarian follicles develop from a reserve of quiescent primordial follicles constituted early in life. During the female life, this ovarian reserve is progressively exhausted by the asynchronous activation of follicles for growth (Gougeon, 1996; McGee and Hsueh, 2000; Monget et al., 2012; Monniaux et al., 2014). Each primordial follicle is composed of a germ cell named oocyte, surrounded by a single layer of about 15 flattened resting somatic cells named granulosa cells. When a primordial follicle is activated by metabolic or hormonal cues coming from the ovarian cortex, its granulosa cells take a cuboidal shape and activate in turn the awakening of the oocyte; this activation process gives rise to a primary follicle consisting of a growing oocyte surrounded by one layer of proliferating granulosa cells (Zhang et al., 2014). Thereafter, multiple layers of proliferating granulosa cells develop around the oocyte which further enlarges. In the pre-antral

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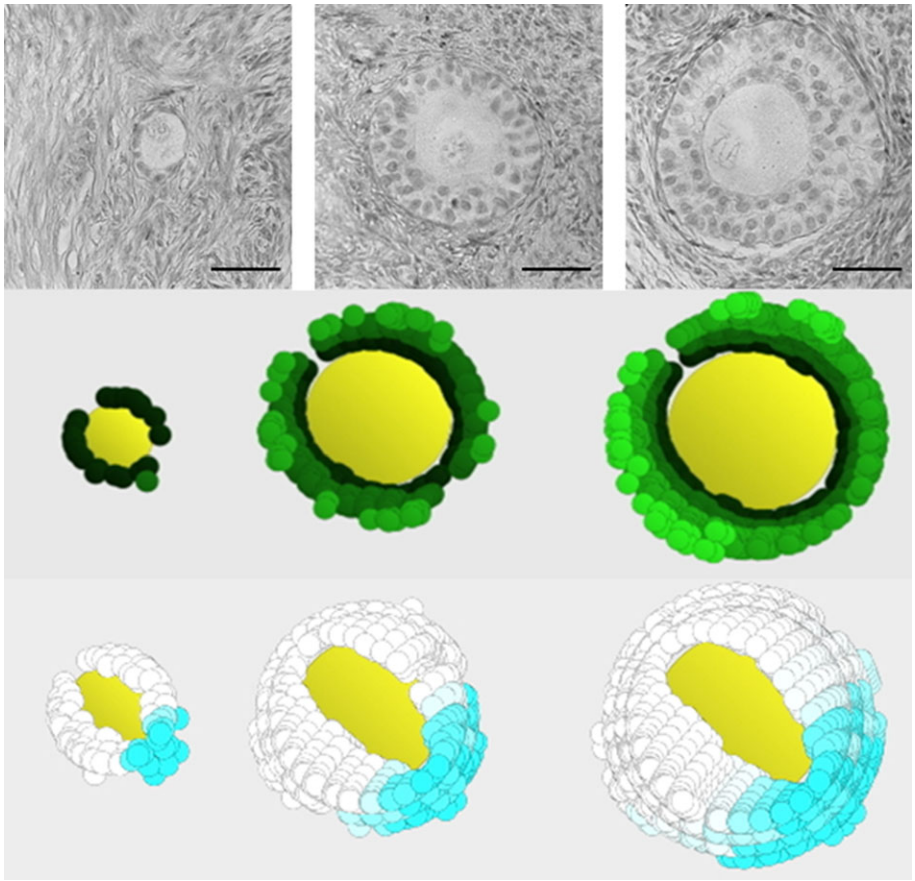
Key words: Cell cycle, Follicle, Germ cell, Mathematical model, Ovary.
Abbreviations: BMP15, bone morphogenetic protein 15; FSH, follicle-stimulating hormone; GDF9, growth differentiation factor 9; KITLG, KIT ligand; LH, luteinising hormone.

Figure 1 | Morphogenesis of small pre-antral follicles

Top panels: histological appearance of growing follicles, from the primary (on the left) to the 3–4 granulosa cell layer (on the right) stages. The oocyte is the big cell at the centre of the follicle. Bar = 50 μm. Centre panels: 3D-like views of microscopic outputs of the model dedicated to the study of follicular morphogenesis (Clément et al., 2013b). Part of the mass of proliferating granulosa cells (small green cells) has been removed to visualise the oocyte (big yellow cell). Bottom panels: 3D-like views of mesoscopic outputs showing a clonal territory (blue cells) in the population of proliferating granulosa cells surrounding the oocyte (big yellow cell). In the clonal territory, the intensity of blue staining varies according to the proportion of cells descending from the same ancestor cell. The temporal changes of follicular morphogenesis are illustrated by simulation outputs which can be visualised in a movie (Film2b.avi) of Appendix C.



follicle made up of three to six granulosa cell layers, a vascularised theca differentiates around the granulosa tissue. At this stage, small extracellular cavities filled with follicular fluid derived from the thecal vasculature begin to form in the granulosa (Rodgers and Irving-Rodgers, 2010). In the small antral follicle (of 250–300 μm diameter in all mammals), the cavities merge to form a single central cavity named antrum which separates the population of proliferating granulosa cells into two main groups, the cumulus cells associated with the oocyte and the mural granulosa cells lining the follicular wall. Antral follicle growth is characterised by the rapid enlargement of the antrum, whereas the mural granulosa cells of the follicle undergo final differentiation, becoming highly estrogenic and LH (luteinising hormone) responsive, and loose concomitantly their proliferative activity (Monniaux et al., 1997). In the antral follicle, the oocyte, after completing its growth, acquires progressively the competence to resume meiosis and ensure normal development of the embryo after fertilisation. The main stages of follicular development are illustrated in Supplementary Figure 1 of Appendix A.

During the so-called basal follicular development, the primary, pre-antral and small antral follicles are little responsive to the pituitary gonadotrophins FSH (follicle-stimulating hormone) and LH. This slow developmental process (which lasts several weeks in rodents and several months in large animals and humans) is controlled by a privileged dialogue between the oocyte and its surrounding granulosa cells. The dialogue operates on the basis of exchanges of small molecules through gap junctions, and the reciprocal action of cytokines and growth factors produced specifically by one of the two cell types (the oocyte or granulosa cell) and acting on the other when present in its vicinity (Kidder and Mhawi, 2002; Otsuka and Shimasaki, 2002; Thomas and Vanderhyden, 2006).

In contrast, the terminal phase of antral follicle development is highly dependent on gonadotrophin supply and is achieved in a few days, within an ovarian cycle. It is a high "risk phase" for the growing follicle which can arrest its development and regress by a physiological process named atresia, if the endocrine and paracrine environment is inadequate for the differentiation stage of its granulosa cells. At each ovarian cycle, the number of ovulations is the result of a selection process in a cohort of simultaneously developing follicles, which is finely regulated by endocrine loops between the ovaries and hypothalamo–pituitary complex (Scaramuzzi et al., 2011).

The granulosa cells are the cellular units orchestrating the development of follicles. During the basal phase of follicular development, their proliferative activity, controlled by tight interactions with the oocyte, drives the growth and shaping of the follicle. In the course of follicular growth, they become gradually more and more responsive to FSH. During the terminal phase of follicular development, the granulosa cells experience a vulnerability phase in which their fate (proliferation, differentiation towards more oestrogenic capacity, or death by apoptosis) is strictly dependent on their FSH environment. This FSH-controlled cell fate will determine the follicle's fate, namely further development towards ovulation or regression by atresia. Putting these observations together, we chose to design modelling approaches centred on the granulosa cell for both the basal and terminal phases of follicular development. These approaches have been developed in sheep, a species of agronomical interest in which data of granulosa cell kinetics, follicular growth (assessed by ovarian ultrasonography scanning) and endocrine time series of FSH and ovarian hormones (oestradiol, inhibin) are available. Moreover, in contrast to rodents, this species presents interesting similarities with the human species for the duration of follicular development, the length of ovarian cycle and the numbers of ovulations.

Follicular morphogenesis: An example of interactions between germ and somatic cells

Cell-based model of follicular morphogenesis

During basal follicular development up to antrum formation, follicular growth operates through an increase in the oocyte size concomitant with the proliferation of its surrounding granulosa cells. In sheep, the oocyte diameter increases 2.8-fold, the granulosa cell population doubles some seven to eight times and the number of granulosa cell layers increases from one to six cell layers before antrum formation (Lundy et al., 1999). The time taken to complete this process varies between 50 and 150 days with very little follicular atresia (Scaramuzzi et al., 2011).

We have developed a spatio-temporal model explaining how the interactions between the oocyte and its surrounding granulosa cells contribute to build a pre-antral follicle, and giving a description of follicular morphogenesis on a cellular and mechanistic basis (Clément et al., 2013b). As the number of granulosa cells is low (less than 20 cells in primary follicles and about 4000 cells in large pre-antral follicles), we chose an individual-based model giving a stochastic and discrete description of follicle development. Each granulosa cell is an individual represented by its age and location in a three-dimensional space. Granulosa cells are subject to both mitosis and displacement events, but there is neither cell death nor quiescence. The general law of evolution of the cell population operates as a counting process, which registers all the events of cell division and displacement affecting the population of granulosa cells. The model is multi-scale in nature since different outputs can be obtained at the individual cell (microscopic) scale (cell location in space, cell age), but also at the semi-local (mesoscopic) scale of cell sub-populations (cell layers and clonal lineages) and at the global (macroscopic) scale of the whole follicle (oocyte and follicle diameters, total cell number).

The model is built on the biologically based hypotheses that granulosa cell proliferation is controlled by factors of the bone morphogenetic protein (BMP) family (BMP15; growth differentiation factor 9, GDF9) specifically secreted by the oocyte, and that oocyte growth is, in turn, under the control of granulosa cell-derived factors such as KIT ligand (KITLG) (Otsuka and Shimasaki, 2002; Thomas and Vanderhyden, 2006). From these assumptions, both the average cell cycle duration of each granulosa cell and the effect of each granulosa cell on oocyte growth depend on the cell distance from the surface of the oocyte.

In this model, the proliferation of granulosa cells driven by oocyte-derived BMP factors is illustrated by the equation:

$$b(t) = 1 - \exp^{-A_k(t)/\lambda_i}$$

$$X_k(t) = (i,\ j)$$

where $b(t)$ is the instantaneous rate of division for cell k at time t, $A_k(t)$ is the age of cell k (time elapsed since its last division), $X_k(t)$ is the location of cell k (the radial distance i corresponding to the layer number and the tangential distance j corresponding to the angular location within a cell layer), and λ_i is related to the average cell cycle duration in cell layer i. The value of λ_i was assumed to increase with the distance of the granulosa cell to the oocyte, in agreement with the lower proliferative activity observed in granulosa cells as they move away from the oocyte and are exposed to increasingly lower concentrations of oocyte-derived mitogenic factors (Da Silva-Buttkus et al., 2008; Gilchrist et al., 2008; Kidder and Vanderhyden, 2010).

The oocyte growth is formulated by a combination of a deterministic law (intrinsic sigmoid-like growth) and a stochastic term driven by KITLG secretion from granulosa cells and acting on the growth rate, as described by the equation:

$$d_O(t) = d_O(0) + \int_0^t (d_O(s))^{\alpha}(1 - d_O(s))^{\beta} \sum_{i \geq 1} \frac{k_i N_i(s)}{\log 2(e)\lambda i} ds$$

where $d_O(t)$ is the oocyte diameter, α and β are oocyte growth parameters, $\kappa_i Ni(t)$ represents the KITLG contribution of layer i to oocyte growth, $Ni(t)$ being the cell number in layer i. The value of κ_i was assumed to decrease when the distance of the granulosa cell to the oocyte increases, illustrating both the dilution of the soluble KITLG form due to diffusion, and the high physiological importance of the membrane-bound KITLG form expressed by the first granulosa cell layer, in close contact with the oocyte (Tajima et al., 1998; Thomas et al., 2008). Each κ_i and λ_i can be deduced through an appropriate recurrence function from the values κ_1 and λ_1 in the first layer.

The model gives a realistic description of the follicular growth and morphogenesis (Figure 1). The granulosa builds as a compact tissue around the oocyte, and the outer contour of the follicle can be more or less regular depending on the degree of filling of the outside layer, in agreement with histological observations. Moreover, the model supports the previously proposed hypothesis that follicles are constructed by the radial proliferation of granulosa cell clones (Telfer et al., 1988; Boland and Gosden, 1994). An example of clonal territory formed from a given progenitor cell is shown (blue cells) in Figure 1.

Genetic control of follicular morphogenesis

In sheep and mouse, some natural or experimental mutations are known to affect the morphogenesis of pre-antral follicles. Our mathematical model can help to study the effects of these mutations on the balance between oocyte growth and granulosa cell proliferation. In sheep, the *FecX*^{I} Inverdale mutation in the *BMP15* gene is known to impair the production of biologically active BMP15 (Galloway et al., 2000), whereas the *FecB*^{B} Booroola mutation in the gene encoding the receptor BMPR1B attenuates BMP signalling in granulosa cells, due to a lower sensitivity of the cells to BMP factors (Mulsant et al., 2001; Fabre et al., 2003; Estienne et al., 2015), associated with a lower production of BMP15 by the oocyte (Crawford et al., 2011). In the ovaries of both *FecX*^{I} and *FecB*^{B} homozygous carrier ewes, follicles contain abnormally large oocytes, and the most severe phenotype is observed in the *FecX*^{I}/*FecX*^{I} ewes which are infertile, due to the arrest of follicular growth at the primary follicle stage (Braw-Tal et al., 1993; Cognié et al., 1998; Wilson et al., 2001). This latter phenotype is reminiscent of that of mice in which the *Gdf9* gene (encoding a Bmp15-homolog oocyte-derived factor) has been deleted (Dong et al., 1996),

Figure 2 |

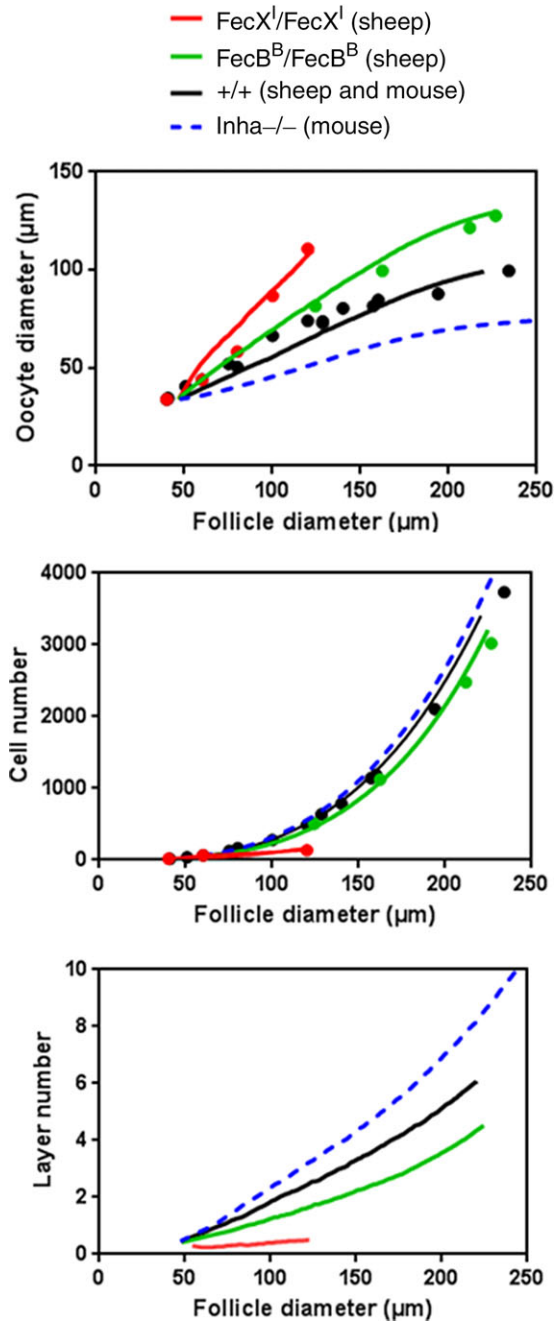


Figure 2 | Genetic control of the balance between oocyte growth and granulosa cell proliferation during the growth of small pre-antral follicles in sheep and mouse

The lines depict different simulation outputs of the model dedicated to the study of follicular morphogenesis (Clément et al., 2013b). Oocyte growth (oocyte diameter in the top panel) and granulosa cell proliferation (total cell number in the center panel and number of cell layers in the bottom panel) are expressed in function of follicular diameter. The simulation outputs reproduce the imbalance between oocyte growth and granulosa cell proliferation associated with some mutations: abnormally large oocytes in follicles from *FecB^B*/*FecB^B* (green lines) and *FecX^I*/*FecX^I* (red lines) ewes, and abnormally small oocytes in follicles from *Inha*-/- (blue dashed lines) mice. In the severe *FecX^I*/*FecX^I* ovarian phenotype, follicular growth is arrested before filling the first granulosa cell layer, leading to sterility of the mutant ewes. Data sets of wild-type (*+/+*, black points), *FecB^B*/*FecB^B* (green points) and *FecX^I*/*FecX^I* (red points) ewes, obtained from Braw-Tal et al. (1993), Lundy et al. (1999) and Wilson et al. (2001), are represented in the top and center panels. No truly quantitative data sets are available for *Inha*-/- mice (Myers et al., 1999).

and experiencing a secondary up-regulation of *Kitlg* in granulosa cells (Elvin et al., 1999). In contrast, the development of multi-layered follicles containing small oocytes in the inhibin-deficient *Inha*-/- mice is associated with the down-regulation of *Kitlg* in granulosa cells (Myers et al., 2009). Using our mathematical model, simulations based on a high value of κ_1 or of the ratio κ_1/λ_1 give rise to follicles with abnormally large oocytes, mimicking the effects on follicular development of the *FecB^B* and *FecX^I* mutations in sheep and of the *Gdf9* deletion in mouse. Conversely, simulations based on a low-ratio κ_1/λ_1 give rise to follicles with abnormally small oocytes, mimicking the effects of *Inha* deletion in mouse (Figure 2). Altogether, the biological data and the simulation results strongly suggest that the balance between the activation level of the BMP and KITLG signalling pathways drives follicular morphogenesis in the early stages of follicular development.

Our model can help to explore the mechanisms underlying the balance between the dynamics of oocyte growth and granulosa cell proliferation in other situations leading to high or low fertility. Imbalance cases can result from natural or experimental mutations in animals, as shown above, but they can also be associated with ovarian pathologies in human. For instance, abnormal follicular morphogenesis has been reported in women suffering from the polycystic ovarian syndrome (Stubbs et al., 2007), but its underlying mechanisms have not yet been investigated.

Follicular selection for ovulation: Cellular mechanisms and hormonal control

Biological knowledge on granulosa cell dynamics during terminal follicular development

At the time when the small antral follicle (with a diameter between 1 and 2 mm diameter in sheep) enters the terminal phase of follicular development, its granulosa cells proliferate actively. The proportion of proliferating cells amongst the whole cell population, also called growth fraction, is the highest and about 75% (Pisselet et al., 2000). About five cell doublings separate the small antral from the preovulatory follicle (with about 6 millions of granulosa cells and 7 mm diameter in sheep), and the succession of cell cycles is accompanied by progressive functional changes in the cell descendants. During this period of about eight days, the proliferation rate of the granulosa cells slows down by gradual exit of cells from the cell cycle, whereas cells become more and more responsive to FSH (in term of FSH-induced cAMP production) and express increasing amounts of inhibin and cytochrome P450 family 19 subfamily A member 1 (CYP19A1), the key enzyme for oestradiol production. FSH orchestrates these processes by both enhancing cell proliferation and promoting cell differentiation. The final differentiation of the granulosa cells involves their FSH-induced endowment of LH receptors in follicles larger than 3 mm diameter. Thereafter, granulosa cells completely stop proliferating and become highly steroidogenic and exquisitely sensitive to LH (Monniaux et al., 1997; Fortune et al., 2001; Scaramuzzi et al., 2011). The main changes in granulosa cell activities during follicular development are illustrated in Supplementary Figure 2 of Appendix A.

During terminal follicular development, the granulosa cells turn progressively from a FSH-responsive to a FSH-dependent maturation stage, in which they enter apoptosis if their FSH requirement is not fulfilled. It is assumed that the propensity of granulosa cells to enter apoptosis is maximal when they achieve their last proliferation cycle and are highly responsive to FSH, but not yet to LH. When the granulosa cells cross this 'vulnerability window', their survival fully depends on FSH fluctuations in their environment. Considering the whole follicle, the transit time of its granulosa cells through this phase of vulnerability and the adequacy of locally bioavailable FSH levels during this transit will determine the cumulative cell loss suffered by the follicle and orientate its trajectory towards ovulation or atresia.

Cell-based model of follicular development

We have designed dynamic models giving a continuous and deterministic description of follicle development, adapted to high numbers of cells (between hundred thousand and millions of cells per follicle) and based on the dynamical repartition of granulosa cells into different cell states (Clément et al., 1997; Clément, 1998; Clément et al., 2002; Echenim et al., 2005; Michel, 2011; Aymard et al., 2012; Clément et al., 2013a; Clément and Monniaux, 2013). In the most complex model, the structuring of the granulosa cell population has been defined in a functional 2D space, where the cellular age (in abscissa) refers to the position within the cell cycle, whereas the maturity level (in ordinate) accounts for cell differentiation level and sensitivity to FSH (Figure 3, top panel). In this formalism, the spatial domain is divided into sub-domains corresponding to different cellular phases: the phase G1 of the cell cycle, during which cells are sensitive to FSH, the aggregation of phases S, G2 and M (phase SM), during which cells are insensitive to FSH and committed to mitosis, and the phase D (differentiation), during which cells having exited the cell cycle are sensitive to FSH for maturation. An additional sub-domain, situated at the boundary between the proliferation and the differentiation domains, corresponds to the vulnerability window (represented as a hatched area in Figure 3), during which cells can enter apoptosis due to FSH deprivation (except when they are in the SM phase, after the restriction point).

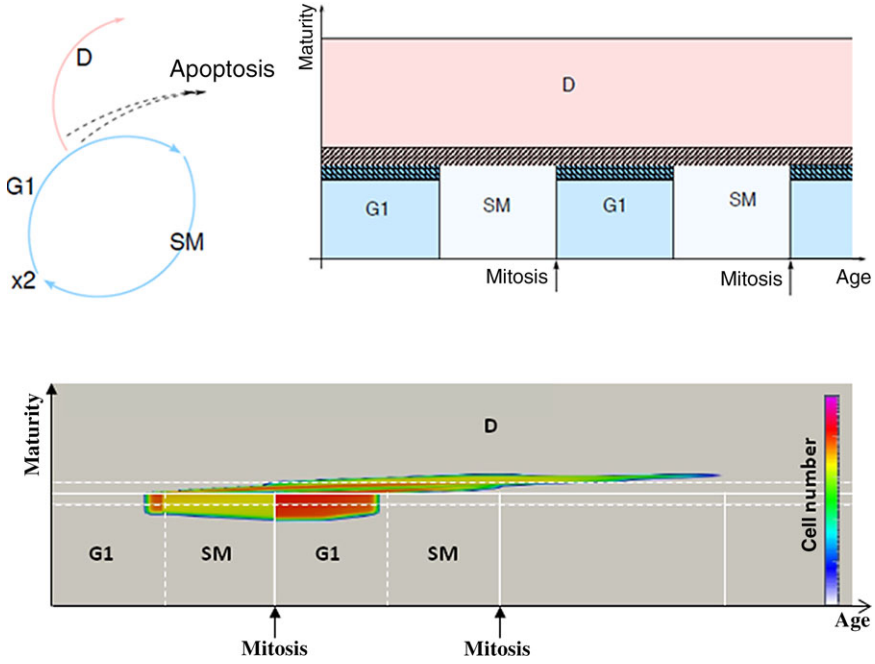
The granulosa cells composing a follicle are spread in the functional domain according to their position within or outside the cell cycle, and their maturity level. The dynamics of the cell density $\Phi_f(a,\gamma,t)$ of the follicle f in the age-maturity domain are described by the following master equation:

$$\frac{\partial \phi_f}{\partial t} + \frac{\partial g_f(a,\gamma,u_f(t))\phi_f}{\partial a} + \frac{\partial h_f(a,\gamma,u_f(t))\phi_f}{\partial \gamma} = -\lambda(a,Y,U(t))\phi_f$$

where the variables a and γ correspond, respectively, to the age and maturity of the follicular cells. The expressions of the ageing and maturation velocities, respectively, $g_f(a,\gamma,u_f(t))$ and $h_f(a,\gamma,u_f(t))$, as

Figure 3 | Functional domain for structured granulosa cell populations and microscopic simulation output of the model dedicated to the study of terminal follicular development

Top panels: the population of granulosa cells in a follicle can be subdivided in sub-populations of proliferating cells, differentiated cells having exited the cell cycle, and cells committed to apoptosis, under the control of FSH levels. A functional domain describing the structuring of cell populations has been defined with cell age in abscissa and cell maturity in ordinate (Echenim et al., 2005; Clément and Monniaux, 2013). Proliferating cells (blue arrows of the cell cycle and blue G1 and SM sub-domains of the spatial domain) can either continue proliferating, or exit the cycle during the G1 phase and enter a differentiated stage (pink arrow of the cell cycle and pink D subdomain of the spatial domain). They can also be committed to apoptosis (black dashed arrows) by FSH deprivation when they cross a vulnerability window (hatched area of the spatial domain) located at the boundary between the proliferation and differentiation sub-domains. Bottom panel: simulation output of the model, illustrating an example of cell repartition in the functional domain. The passage of cells through the SM-G1 interface has resulted in doubling of the cell density. The cell population spread in the D sub-domain includes cells having exited the cell cycle during the G1 phase of the current and former cell cycles. A movie (Gmovie_fol0.mov) provided in Appendix C illustrates the temporal changes of cell states in the functional domain during follicular development.



well as that of the cell loss rate through apoptosis $\lambda(a,\gamma,U(t))$, vary according to the spatial location of the cell in the domain; their mathematical formulation is provided in Appendix B. These terms are subject to the control functions $u_f(t)$, which reflects the bioavailable level of FSH in follicle f and $U(t)$, which represents plasma FSH. They can be interpreted at the intracellular level since the commitment of granulosa cells to proliferation, differentiation or apoptosis is controlled by FSH-induced cAMP dynamics (Clément et al., 2001). An output of the model (snapshot in the bottom panel of Figure 3) illustrates the repartition of the granulosa cells of a growing follicle into the different functional sub-domains at a given time of the simulation.

On the follicle scale, interesting model outputs are the growth fraction and mitotic index (percentage of mitoses) of the granulosa cell population, the number of cells lost by apoptosis, and the total cell number. In addition, the follicular maturity corresponds to the follicle production of inhibin and oestradiol, that is associated with LH responsiveness, ensures its ability to ovulate and weights its contribution into the endocrine feedback exerted by the ovaries onto the pituitary gland and hypothalamus. The total cell number and follicular maturity correspond,

respectively, to the zero-order and the first-order moments of the cell density, defined by:

$$m_f^0(t) = \iint \phi_f(a, \gamma, t) da\, d\gamma$$

$$m_f^1(t) = \iint \gamma \phi_f(a, \gamma, t) da\, d\gamma$$

The changes of these outputs with time give an in-depth cellular and functional description of the dynamics of follicle development towards ovulation or atresia.

Follicle competition for ovulation

From the available biological data, the ovulatory follicle(s) is (are) selected within a cohort of antral growing follicles, also called a follicular wave, whose emergence and growth are triggered by relatively high blood concentrations of FSH (Ireland et al., 2000; Ginther et al., 2001; Scaramuzzi et al., 2011). Each follicle of the cohort secretes increasing amounts of inhibin and oestradiol, relative to its cell number and cell maturity level, during its development. The cumulated contributions of all growing follicles to the release of oestradiol and inhibin by the ovaries lead to a drop in FSH secretion by the pituitary gland, and the follicles producing the highest hormonal outputs take progressively the control of FSH secretion. The drop in plasma FSH concentrations is at the source of the selection process for ovulation occurring within the follicle population. A hierarchy based on the functional heterogeneity amongst follicles of the cohort is established progressively, and the follicles become atretic when FSH concentrations fall below the threshold needed to sustain their development. In a mono-ovulating species (such as human, cattle, horse, sheep), only the most mature follicle can survive in the presence of decreasing concentrations of FSH. This follicle has acquired the most developed vascularisation and its granulosa cells are the first to become LH responsive, before those of all other follicles in the cohort; it completes its development thanks to LH support until the pre-ovulatory stage, whereas the other follicles degenerate by atresia.

Our cell-based model describing follicular development takes into account the hormonal feedback loop involving the growing ovarian follicles and pituitary gland (for review: Clément and Monniaux, 2013). The ovarian maturity is defined as the sum of the maturities of all follicles in the cohort:

$$M(t) = \sum_f m_f^1(t)$$

The global control variable $U(t)$ represents FSH concentrations in plasma, and is a decreasing sigmoidal function of $M(t)$. The local control $u_f(t)$ represents FSH bioavailable intra-follicular concentrations, and is proportional to $U(t)$, with a rate evolving as an increasing sigmoidal function of $m_f^1(t)$, that takes into account the increase in vascularisation and bioavailable FSH during follicle maturation. The mathematical formulations of $U(t)$ and $u_f(t)$ are provided in Appendix B.

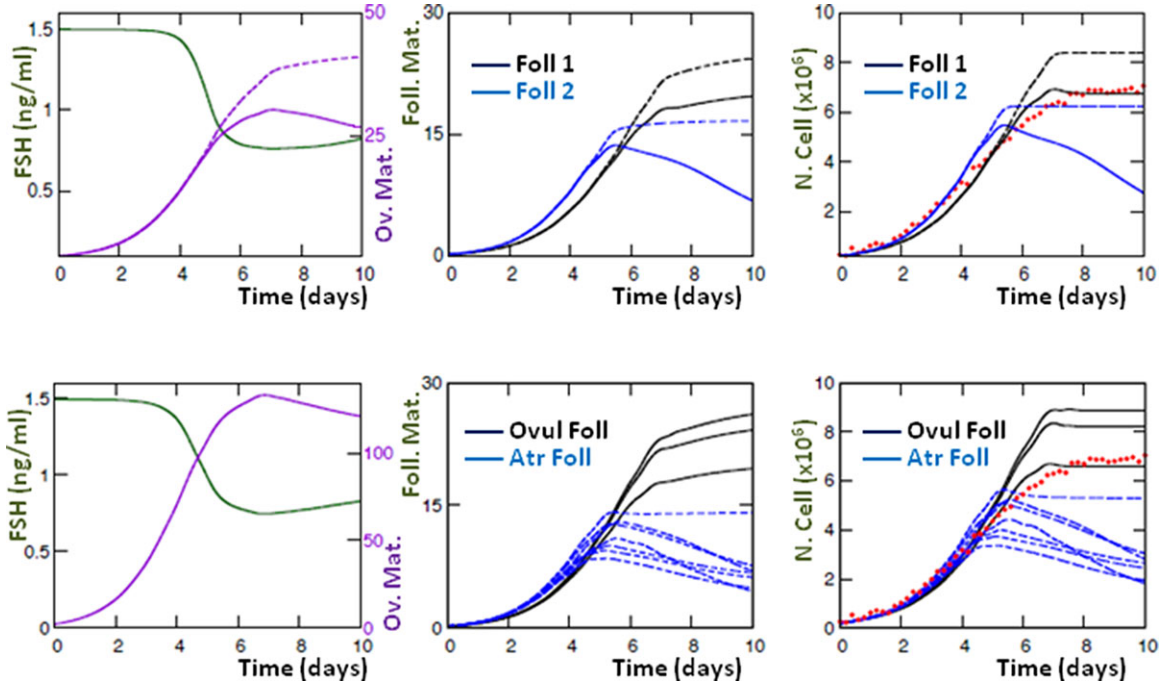
This model is clearly multi-scale in nature. Outputs can be obtained at two embedded microscopic scales, *i.e.* the granulosa cells and the granulosa cell populations, and two embedded macroscopic scales, one considering the follicle (size, cell number, maturity) as an individual, the other the ovarian hormonal release integrating the contribution of all granulosa cells in the cohort of growing follicles. In this model, the separation between the individual trajectories of follicles lies upon differences in the distribution of the cell populations making up each follicle amongst the phases G1, SM and D of the functional domain. These differences result in differential timings in the maturation steps marking follicular development (as the complete switch to differentiation or escape from vulnerability window) and contrasted total cell numbers and maturity levels at the time of ovulation, ultimately determining the fate of each follicle, *i.e.* ovulation or atresia.

Calibration of interacting ovulatory and atretic trajectories

The complexity of our mechanistic model coupling cell kinetics with population dynamics, associated with the scarcity of quantitative and kinetic biological data (particularly at the microscopic scale), raises challenging questions to calibrate the model parameters, that we have recently addressed (Aymard et al., unpublished data). Combining data on follicular growth (changes in follicular diameter with time assessed by ultrasonography (Ravindra et al., 1994) or histology (Turnbull et al., 1977)) with data on granulosa cell numbers according to the follicular

Figure 4 | Macroscopic simulation outputs of the model dedicated to the study of terminal follicular development (unpublished data from M. Postel and F. Clément)

The panels illustrate different outputs as functions of time for a cohort of two follicles (top panels) and 10 follicles (bottom panels). The ovulatory follicles are defined as follicles having reached total cell numbers and maturity levels sufficient for ovulation, both being specific to each ovine breed or line. Ovulation is triggered when the ovarian maturity (which is the sum of the maturities of the follicles composing the cohort) is above a threshold. A high ovarian maturity level is reflected in blood by high oestradiol concentrations, which act upon the hypothalamo-pituitary system and can trigger the GnRH (gonadotrophin releasing hormone)-induced pre-ovulatory LH (luteinising hormone) discharge when they exceed a threshold. At this time, only the most mature follicles, bearing LH receptors on their granulosa cells, can ovulate in response to this LH discharge. Left panels: plasma FSH and ovarian maturity. Center panels: follicular maturity (expressed in arbitrary units), corresponding for each follicle to its capacity of production of oestradiol (associated with its responsiveness to gonadotrophins). Right panels: number of granulosa cells per follicle. The red points correspond to an experimental data set elaborated from different bibliographic sources (Clément et al., 1997). In the top panels, the dashed lines correspond to the follicular trajectories when the apoptosis rate is deactivated. In the bottom panels, the solid black lines correspond to the ovulatory follicles and the dashed blue lines to the atretic ones, whose final maturity and cell number are insufficient for ovulation.



diameter (Tsonis et al., 1984), it has been possible to build a data set relating the cell number to time (*i.e.* follicle age) for the trajectory of an ovulatory follicle in sheep (Clément et al., 1997). This data set was used as a basis for the parameter calibration strategy, to define both the time (and corresponding cell number) when the follicles switch from a FSH-responsive to a FSH-dependent status, and the time when they are selected as future ovulatory follicles. The pattern of FSH changes during the growth of the follicular cohort was assessed in a more direct way from time series of FSH plasma concentrations along the ovarian cycle (Ravindra et al., 1994; Toosi et al., 2010). Using these data, the specific assumptions of the model and multi-objective functions designed along the different calibration steps, it was possible to obtain distinct sets of parameters discriminating the ovulatory trajectories from the atretic ones in a FSH-poor environment (Aymard et al., unpublished data).

An example of simulation output for an ovulatory/atretic pair of follicles is presented in the top panels of Figure 4 (solid lines). To visualise the contribution of the apoptotic process in the dynamics,

the same pair is also followed in the case when the apoptosis rate is deactivated (dashed lines); the selection is then not operated and the former atretic follicle becomes able to ovulate. This result agrees with the biological concept that there is no predestination of the follicle fate, but that the cell sensitivity to the hormonal control is at the source of the selection. The bottom panels of Figure 4 illustrate another example of simulation outputs with a cohort of 10 follicles. In this simulation, all follicles start with the same normalised cell number, but with random perturbations (in the $\pm 10\%$ range) of the values of the parameters which govern the maturation velocities. The follicles are sorted amongst ovulatory follicles (black solid lines) and atretic ones (dashed blue lines). At the ovulation time (about eight days, when the cumulated ovarian maturity has reached a threshold level sufficient to trigger ovulation), three follicles of the cohort are able to ovulate.

The multi-scale model of follicular development is a useful tool to study the mechanisms regulating the number of ovulations (ovulation rate) in different breeds and lines of sheep. In this naturally mono-ovulating species, the presence of specific mutations affecting factors of the BMP family or their receptors has been associated with the occurrence of multiple ovulations and the advancement of follicular maturation, so that follicles ovulate at a smaller size, compared with follicles of wild-type ewes (McNatty et al., 2005a, 2005b; Fabre et al., 2006). The physiological mechanisms responsible for this ovarian phenotype have not been fully understood up to now. Using the multi-scale model through intensive simulations with random selection of parameter values, one can expect to recover for each parameter a distribution compatible with the ovulation number and ovulatory size of follicles for each genetic model. For instance, the simulation outputs depicted in the bottom panels of Figure 4 show that the final cell mass and maturity of the ovulatory follicles are modulated by small perturbations in parameters affecting the ageing and maturation velocities. Elucidating the mechanisms resulting in a given ovulation number is not a trivial question since the poly-ovulatory strategies involve different mechanisms acting on the follicle populations (*e.g.* size of the cohort at terminal recruitment, rate of selection) or individual follicles (*e.g.* ovulatory size, final cell number, cellular steroidogenic potential) and endocrine feedbacks (sensitivity of pituitary or hypothalamic cells to ovarian hormones). The model helps us to investigate these mechanisms and especially (i) the compromise between proliferation and differentiation that tunes and possibly optimises the final cell number and corresponding maturity at ovulation time (Clément, 1998; Clément et al., 2013a); (ii) the quite antagonistic constraints weighing on the atresia rate, that should both limit cell loss in ovulatory follicles and penalise sufficiently the atretic ones to deplete their cell content; and (iii) the degrees of freedom operating at the level of the ovaries, pituitary gland or hypothalamus (Echenim et al., 2005). The corresponding qualitative and semi-quantitative scenarios would be substantiated further if the model could be fuelled with proper combinatory data sets describing both the atretic and ovulatory trajectories, which has remained beyond reach on the experimental ground up to now. In the same spirit, subject to data availability, the model could be extended to other species characterised by one (human, cattle, horse) or multiple ovulations (rodents, pigs) at each ovarian cycle, and to pathological situations of dysovulation or anovulation.

Conclusion

The multi-scale and dynamic models that we have designed to study follicular development are based on the embedding of physiological mechanisms into the cell scale. These mechanistic models have been built on biological hypotheses and concepts and result in mathematical objects that raise challenging numerical (Aymard et al., 2013) and theoretical questions (Shang, 2013). Even if the available quantitative data are scarce and not truly kinetic, the design of precise biological specifications from combined literature sources enabled us to constrain the quantitative outputs on the different scales and calibrate parameters entering the microscopic functions from macroscopic observations.

Despite their difficult calibration, these models are useful tools for addressing ovarian physiopathological situations. Moreover, they can be proposed as generic modelling environments to study various developmental processes and cell interaction mechanisms. The model of follicular morphogenesis based on the molecular dialogue between two neighbouring cell types takes into account the secretion, diffusion and action of factors in a spatio-temporal

formalism which could be applied to organogenesis in embryo and fetus, or tumour development. In the model dedicated to terminal follicular development, we have addressed very generic processes of cell developmental biology such as the dynamical repartition of cell populations into different states (namely, proliferation, differentiation and apoptosis), the regulation of cell cycle progression and exit towards apoptosis or differentiation by an extracellular factor, and the feedback of cell populations on their own renewal. The simulations enable real-time monitoring of biological markers such as the growth fraction and mitotic index, and the distribution of cells within the cell cycle. At this stage, the information provided by the models is richer than what can be obtained *in vivo* or in primary cell cultures. Yet, there is hope that the adaptation of new methodologies (Sakaue-Sawano et al., 2008; Hoppe et al., 2014) to the physiological context of a tissue will help to match the *in silico* time-lapse monitoring of cell fates or cell populations to *in vivo* measures.

Conflict of interest statement

The authors have declared no conflict of interest.

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