Human ovarian follicular development: From activation of resting follicles to preovulatory maturation

Croissance folliculaire dans l’ovaire humain : de l’entrée en croissance du follicule primordial jusqu’à la maturation préovulatoire

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Résumé

En intégrant les données morphologiques et endocrinologiques disponibles ainsi que les effets biologiques des diverses molécules synthétisées par le follicule, nous proposons une vision dynamique de la croissance folliculaire dans l’ovaire humain. La folliculogenèse débute par l’entrée en phase de croissance des follicules au repos, un processus où le système kit joue un rôle central. Il faut plusieurs mois pour qu’un follicule en début de croissance atteigne le stade préantral (0,15 mm) et 70 jours supplémentaires pour qu’il atteigne une taille de 2 mm. La croissance de petits follicules est finement régulée par des interactions entre la follicle-stimulating hormone (FSH) et les diverses molécules synthétisées par la granulosa, la thèque interne et l’ovocyte. Lorsqu’ils deviennent sélectionnables, les follicules deviennent sensibles aux variations cycliques de FSH en termes de prolifération des cellules de la granulosa (CGs). Au début de la phase folliculaire, le follicule sélectionné se développe très rapidement et synthétise de l’œstradiol. Toutefois, la production totale de stéroïdes reste modérée. À partir du milieu de la phase folliculaire, le follicule préovulatoire synthétise des quantités croissantes d’œstradiol, puis après la décharge ovulante de grandes quantités de progestérone. La capacité de réponse des CGs aux gonadotrophines, et plus particulièrement à luteinizing hormone (LH), est alors maximale. LH induit la dissociation de la granulosa et l’expansion du cumulus ainsi que la maturation nucléaire de l’ovocyte. En conclusion, on peut dire qu’au cours de son développement, la capacité du follicule à répondre aux gonadotrophines augmente en réponse à des facteurs locaux agissant de façon autocrine et/ou paracrine.

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Mots clés : Ovaire ; Follicules ; Ovocyte ; Granulosa ; Thèque interne ; Facteurs de croissance ; Gonadotrophines ; Stéroïdogenèse

Abstract

By integrating morphometrical and endocrinological data, as well as biological effects of various molecules synthesized by the human follicle, we propose a dynamic view of the follicle growth within the human ovary. Folliculogenesis starts with entry of resting follicles into the growth phase, a process where the kit system plays a key role. Several months are required for a new growing follicle to reach the preantral stage (0.15 mm), then 70 additional days to reach the size of 2 mm. Early growing follicle growth is regulated by subtle interactions between follicle-stimulating hormone (FSH) and local factors produced by theca and granulosa cells (GCs), as well as the oocyte. From the time they enter the selectable stage during the late luteal phase, follicles become sensitive to cyclic changes of FSH in terms of granulosa cell proliferation. During the early follicular phase, the early selected follicle grows very quickly and estradiol is present in the follicular fluid. However, the total steroid production remains moderate. From the mid-follicular phase, the preovulatory follicle synthesizes high quantities of estradiol, then after the mid-cycle gonadotropin surge, very large amounts of progesterone. At this stage of development, the responsiveness of the follicle to gonadotropins is maximum, especially to luteinizing hormone (LH) that triggers granulosa wall dissociation and cumulus expansion as well as oocyte nuclear maturation. Thus, as the follicle develops, its responsiveness to gonadotropins progressively increases under the control of local factors acting in an autocrine/paracrine fashion.

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Keywords: Ovary; Follicles; Oocyte; Granulosa; Theca interna; Growth factors; Gonadotrophins; Steroidogenesis

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1. Introduction

In humans, folliculogenesis starts when resting follicles leave the ovarian reserve and culminates with the production of a single dominant follicle during each menstrual cycle. This process can be divided into four main steps: initiation; early follicle growth; selection of one follicle from a pool of selectable (≥ 2 mm) follicles; and maturation of the preovulatory follicle (Fig. 1). While morphological and dynamic aspects of human follicular growth did not substantially change during the past years, significant progress in the knowledge of factors regulating ovarian function has been made by different techniques, including in vitro studies, transgenic mice models, microarray analyses and phenotypic observations of women bearing spontaneous mutations affecting fertility.

One of the most exciting facts that has recently emerged is that local factors, especially those produced by the oocyte, play a critical role in folliculogenesis, including activation of resting follicles, early growth, and terminal maturation. For example, it has been demonstrated [1] that growing oocytes accelerate follicular development and strongly suggest that the oocyte may be operating as a “folliculogenesis clock”. The use of transgenic mice models has provided evidence that mutations in many genes affect the synthesis of hormones and growth factors and/or responsiveness of hormones and growth factors, which in turn indirectly affect folliculogenesis. The data obtained from transgenic mouse models may provide clues about the reasons for abnormal reproductive conditions in humans, because multiple genes shown to regulate folliculogenesis and fertility in mice have been shown to play a role in human reproduction. However, the data obtained in the mouse must be considered with caution before extrapolation to humans since they may be irrelevant because of strong differences existing between human and mouse folliculogenesis. The array technologies are also very promising tools to identify which genes are either down- or up-regulated during a given step of folliculogenesis. The aim of this article is to remember the changes affecting the follicular tissues during folliculogenesis and to describe the regulations that may be operating inside the primate follicle at the different stages of its development and especially those that may be relevant for future researches to improve fertility in humans.

2. Initiation of follicular growth

2.1. Composition of the ovarian reserve

After birth, the pool of follicles at the resting stage constitutes the ovarian reserve. It includes primordial, transitory and small primary follicles (Fig. 2A, B, C). These follicle subtypes differ in their proportion of flattened and cuboidal granulosa cells (GCs) and in their diameter, due to differences in the number and size of GCs, but they do not differ in mean diameter of their oocyte and its nucleus [2]. They do not express functional gonadotropin receptors [3] and represent between 91 and 98% of the total ovarian follicular population.

2.2. Ageing changes

In humans, as in other mammals, the size of the ovarian reserve decreases drastically with age. At birth, the size of the ovarian reserve varies between individuals. Although follicular counts have been performed in a small number of newborn ovaries, it can be proposed, from both counts [4,5] and mathematical extrapolation [6] that each ovary contains between 250,000 and 500,000 healthy resting follicles. In humans, the rate of follicular depletion might be variable between subjects and accelerates from approximately 38 years of age onward, leading to a stock at menopause estimated between less than 100 [6] and 1000 [7] resting follicles per ovary. The discrepancy between these two studies can be explained by the follicular classification used by the authors, in which transitory and small primary follicles were considered either as resting [6] or growing follicles [7], despite the demonstration that transitory follicles are still resting follicles in rats [8] and cattle [9].

In all mammalian species, follicles leave the stock of resting follicles in a continuous stream, either by apoptosis or by entry into the growth phase.

2.3. Depletion of the pool by atresia

It is now widely accepted that apoptosis, an active energy-consuming process controlled by a number of intracellular proteins, is mainly responsible for attrition of the ovarian reserve. Atresia of resting follicles is very difficult to appreciate because of a very quick disappearance of apoptotic oocytes (Fig. 2E). However, by talking into account the number of growing follicles within the ovaries throughout life [6], it can be estimated that more than 90% of the ovarian reserve escapes by apoptosis mainly during infancy and early adulthood. In most cases, survival or apoptosis results from a balance between expression of survival (antiapoptotic) and pro-apoptotic factors. Among these factors, the proteins bcl-2 and bax likely play a critical role. On the one hand, in bcl-2 deficient mice, decreased numbers of follicles are present after birth [10], while overexpression of bcl-2 leads to decreased follicular apoptosis [11], and, on the other hand, bax deficiency leads to prolongation of ovarian lifespan [12]. However, given the very high number of proteins involved in apoptosis, bax and bcl-2 are probably not the only factors responsible for attrition of the human ovarian reserve. In addition, the factors responsible for the onset of the apoptotic cascade in resting follicles in physiologic conditions remain unknown and, therefore require further very exciting studies.

2.4. Depletion of the pool by initiation of follicular growth

In humans, the follicles enter the growth phase, continuously from fetal life to menopause, when the oocyte nucleus reaches a critical diameter of 19 μm [2]. The precise timing of events leading to activation of resting follicles is not still determined, but multiple experiments [13] and observations showing that folliculogenesis is normal up to the preantral stage in women with follicle-stimulating hormone (FSH) deficiency and in both
FSH-ß subunit and FSH-receptor knock-out (KO) mice [14–16], strongly suggest that luteinizing hormone (LH) and FSH are not directly involved in this process.

The follicles can be blocked at the resting stage for a very long time, up to 50 years in humans. Consequently, one question can be addressed: is follicle quiescence due to the action of a molecule(s) preventing resting follicles from activation?

Anti-Müllerian hormone (AMH) inhibits initiation of follicular growth in neonate rat ovaries [17], an effect that may be due to inhibition of stimulatory factors [18]. In humans, Carlsson et al. [19] reported an inhibitory effect of AMH on initiation in cultured ovarian cortical biopsies, however, this finding was disputed by others [20] who assumed that AMH initiates growth of primordial follicles in the same culture model. Somatostatin (SST), a potent inhibitor of cAMP generation in most epithelial cells [21] is able to inhibit activation of the kit system in the testis [22], and a SST receptor antagonist can increase spontaneous initiation of follicular growth in cultured ovaries from neonatal mice [23]. It has also been observed that mice lacking the transcription factors FOXO3a [24] and FOXL2 [25], which are present in the human ovary, exhibit an accelerated depletion of the ovarian reserve suggesting that these transcription factors inhibit initiation. In humans, a mutation of FOXL2 is associated to premature ovarian failure in patients suffering from blepharophimosis-ptosis epicanthus inversus syndrome (BPES). Interestingly, in one BPES patient, the early growing follicles do not product AMH [26], and in mice lacking FOXL2, AMH expression is absent, suggesting that these transcription factors may maintain follicles at the resting stage via AMH.

In mice displaying spontaneous mutations of steel, the cAMP-responsive [27] gene coding for the kit ligand (KL) [28], follicular growth is more or less blocked at the primary stage. This role of the kit system has been experimentally confirmed by injecting antibodies against c-kit, the receptor for KL, [29] to neonate mice and by culturing neonate rat ovaries in the presence of either KL or antibodies against c-kit [30]. In humans, KL mRNA is present in GCs [31], and c-kit in GCs and theca interna cells (TICs) [32], whereas in the monkey KL and c-kit proteins are present in primordial follicles [33]. Since AMH has recently been shown to downregulate c-kit expression in the rat [18], it appears clearly that the kit system plays a key role, if not central, in initiation of follicular growth.

Other molecules have been reported able to stimulate initiation of follicular growth. A body of evidence argue in favor of a role for neurotrophins, a group of molecules including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophins in initiation of follicular growth [34,35]. These observations are relevant for human reproduction since NGF is produced by both human GCs and TICs [36,37]. The cultured neonatal rat or mouse ovary model was used to show that other proteins, such as basic fibroblast growth factor 2 (FGF2) [38], keratinocyte growth factor (KGF) [39], leukemia inhibiting factor (LIF) [40], insulin [41], bone morphogenetic protein-4 (BMP4) [42], platelet-derived growth factor (PDGF) [43] and activin [44], as well as treatment with androgens in monkeys [45], can stimulate initiation of follicle growth.

Thus, many factors seem to be involved in initiation of follicular growth, but the way by which they act either to inhibit or to stimulate this process remains to be determined. Interestingly, expression of both the c-kit and the FGF receptor (FGFR1) are suppressed by AMH [18] and, in addition, PDGF [43] and FGF2, which is present with most of its receptors in human oocytes and GCs [46,47], might act by stimulating GC-production of KL [38].

Taken together, the available data strongly suggest that the kit system plays the central role in the process by which

Fig. 1. Schematic representation of the four stages of human folliculogenesis showing their duration, the size of follicles and the status of GCs in terms of responsiveness to FSH.
Fig. 2. Human ovarian follicles. A. Primordial follicle, the oocyte is surrounded by flattened GCs (scale bar = 18 μm). B. Intermediary follicle, the oocyte is surrounded by a mixture of flattened and cuboidal GCs (scale bar = 18 μm). C. Small primary follicle, the small oocyte is surrounded by a single layer of cuboidal GCs (scale bar = 18 μm). D. Large primary follicle in which a large oocyte is surrounded by a single layer of cuboidal GCs (scale bar = 20 μm). E. Atretic primary follicle (top right) in which the oocyte has disappeared; a normal primordial follicle (left) is present (scale bar = 30 μm). F. Secondary follicle (scale bar = 40 μm). G. Preantral follicle (class 1, 0.15–0.2 mm) (scale bar = 75 μm). H. High power micrograph of epithelioid theca cells (white arrow) from a preantral follicle (scale bar = 18 μm). I. Follicle with a small antrum (class 2, 0.2–0.4 mm) (scale bar = 100 μm). J. Early antral follicle; the GCs surrounding the oocyte constitute the cumulus oophorus (class 2) (scale bar = 90 μm). K. Small antral follicle (class 3, 0.5–0.9 mm); resting follicles are on right (scale bar = 160 μm). L. Small antral follicle (class 4, 1–2 mm) (scale bar = 300 μm). M. Selectable follicle (class 5, 2–5 mm) (scale bar = 530 μm).
the appearance of typical steroid-secreting cells also referred from the cells of the undifferentiated theca. In the inner part, externa, is composed of cells that do not differ in any respect stratifies and differentiates in two parts. The outer part, the theca and constitute the theca layer. As the follicle enlarges, this theca cells near the basal lamina become aligned parallel to each other differentiating in the blood. At this stage of development, some stroma cells, with two or more complete layers of GCs surrounding the oocyte, which might possess FSH receptors [52,53], starts to produce local factors such as GDF9 and BMP15 that are specific regulators of follicle development. The oocyte growth is sustained by KL up to the preantral stage only in the presence of contact communication with GC [54]. However, the oocyte can control its own growth through GDF9 that downregulates KL expression in the mouse [55], which, in its turn, downregulates BMP15 expression [56]. In the GDF9-KO mouse model, where follicles possess no thecal layer and contain an asymmetrical arrangement of atypical GCs, follicular growth is blocked beyond the secondary stage. This abnormal oocyte growth could be due to an overexpression of KL in the absence of GDF-9 [57] leading, among others to a BMP15 inhibition.

3.3. Theca cell proliferation and differentiation

The mechanisms leading to differentiation of stroma cells surrounding primary/secondary follicles into TICs remain unclear. They may involve the action of GDF-9 [58], KL [59] and epidermal growth factor (EGF) [60], which have been postulated to as epithelioid cells (Fig. 2H). From the time of appearance of epithelioid cells, the secondary follicle is defined as a preantral follicle (Fig. 2G) and constitutes the first class of growing follicles in a classification based on morphological aspect and total number of GCs in each individual follicle [13]. When small fluid-filled cavities aggregate to form the antrum, the follicle becomes early antral (Fig. 2I) and possesses follicular fluid having a composition near that of the blood serum. From this time, the GCs surrounding the oocyte constitute the “cumulus oophorus” (Fig. 2J). Because of the great importance of multiple interactions between the oocyte and follicular tissues [1], the GC-oocyte gap-junctions play a critical role during folliculogenesis as demonstrated by the lack of preovulatory follicles in CX37 null mice [49]. Through accumulation of fluid in the antral cavity and proliferation of GCs and TICs, the follicle progresses at an increasing rate through subsequent stages of development (Fig. 2K, L) until it reaches a size comprised between 2 and 5 mm and becomes a selectable follicle (Fig. 2M). In humans, the time for early growing follicles to reach the preantral stage is not known, but has been estimated around 2 to 3 months, 70 additional days being necessary for preantral follicles to reach a size around 2 mm [50]. This chronology has recently been validated since a preovulatory follicle was observed 5 months after orthotopic autotransplantation of frozen ovarian cortical tissue in a patient suffering from a POF after having undergone a chemotherapy for Hodgkin’s lymphoma [51].

Thus, at the beginning of the follicle growth, the oocyte enlarges, TICs proliferate and differentiate and GCs proliferate.

3.2. Oocyte growth

It is during early follicle development that the oocyte grows at the quickest rate; indeed in humans, its diameter increases from approximately 40 μm in large primary follicles to approximately 100 μm in early antral follicles. Beyond this stage of development, the oocyte diameter increases at a very slow rate to reach approximately 140 μm in the preovulatory follicle. The oocyte, which might possess FSH receptors [52,53], starts to produce local factors such as GDF9 and BMP15 that are specific regulators of follicle development. The oocyte growth is sustained by KL up to the preantral stage only in the presence of contact communication with GC [54]. However, the oocyte can control its own growth through GDF9 that downregulates KL expression in the mouse [55], which, in its turn, downregulates BMP15 expression [56]. In the GDF9-KO mouse model, where follicles possess no thecal layer and contain an asymmetrical arrangement of atypical GCs, follicular growth is blocked beyond the secondary stage. This abnormal oocyte growth could be due to an overexpression of KL in the absence of GDF-9 [57] leading, among others to a BMP15 inhibition.

3.1. Morphological aspects

When follicles enter the growth phase, they enlarge, both by proliferation of GCs and by increase in size of the oocyte. The first stage of follicular growth in humans is the large primary follicle (Fig. 2D). At this stage of growth, a network of gap-junctions, intercellular membrane channels that allow nutrients, inorganic ions, second messengers and small metabolites to pass from cell to cell, appear in the granulosa layer. Each of these channels are composed of connexins (CX). CX43 is the most abundant CX in the ovary and is expressed in the GCs from the start of folliculogenesis, that is arrested at the primary stage in CX43 null mice [48].

Progressively, follicles become secondary follicles, i.e. follicles with two or more complete layers of GCs surrounding the oocyte (Fig. 2F) that are served by one or two arterioles, terminating in an anastomotic network just outside the basal lamina. The physiological importance of this event is emphasized by the fact that the follicle becomes directly exposed to factors circulating in the blood. At this stage of development, some stroma cells near the basal lamina become aligned parallel to each other and constitute the theca layer. As the follicle enlarges, this theca stratifies and differentiates in two parts. The outer part, the theca externa, is composed of cells that do not differ in any respect from the cells of the undifferentiated theca. In the inner part, the theca interna, some fibroblast-like precursor cells assume the appearance of typical steroid-secreting cells also referred...
to act as “granulosa-derived theca cell organizers”. LH has been considered to be the primary hormone regulating the epithelioid differentiation of TICs [61]. However, IGF-I may be acting as differentiation factors for TICs in inducing either LH receptors [62], present in primates in all follicles from the preantral stage [63], or steroidogenic enzymes [64].

In humans, the theca interna is the primary site of androstenedione (A) synthesis, which is the principal aromatizable steroid, whereas testosterone (T) is produced in lesser amounts [65]. The androgen secretion by TICs under LH stimulation appears to result from activity of enzymes, such as cholesterol side chain cleavage (P450scc), 17α-hydroxylase/lyase (P45017α/lyase), and 3β-hydroxysteroid dehydrogenase (3β-HSD). Nevertheless, during basal growth, primate follicles exhibit slight steroidogenic activity as illustrated by a low expression of P45017α/lyase in human TICs (Fig. 4A).

3.4. Granulosa cell proliferation and differentiation

3.4.1. Granulosa cells are weakly responsive to FSH during basal follicular growth

When early growing follicles become vascularized, they are directly exposed to factors circulating in the blood, especially FSH, which is the primary regulator of ovarian folliculogenesis. However, despite the presence of FSH-receptors on GCs, the role of FSH in sustaining early follicular growth remains unclear since these follicles seem to be unresponsive to gonadotropins. Indeed, follicles smaller than 2 mm do not display any change of their GC proliferation in response to cyclic changes of FSH [13], and women undergoing assisted reproductive technology (ART) had fewer number of oocytes obtained than the number of selectable follicles (> 2 mm) detected at ultrasonography at the time when treatment starts [66].

It has been proposed that early growing follicles are FSH-independent since they can reach the selectable stage in the absence of either bioactive FSH [14,16] or FSH receptor [15]. Moreover, in various pathological (hypogonadotropic hypogonadism, hypophysectomy) situations in humans, selectable follicles were observed despite extremely low levels of gonadotropin [13], and, in patients displaying high levels of gonadotropins, because of a partial inactivation of the FSH receptor gene, follicles can grow up to a size of several millimetres [67] but cannot reach the ovulatory size. Taken together, these observations indicate that in presence of very low levels of FSH activity, folliculogenesis can take place until the selectable stage (≥ 2 mm).

Many local factors such as activin A [68], EGF [69], TGF-β [70], FGF2 [71], hepatocyte growth factor (HGF) [71], GDF-9 [72] and BMP-15 [73] have been shown to stimulate GC proliferation in the absence of FSH. Whether these factors, which are present in the monkey or human ovary [33,46,74–79], act in a similar way on early growing human follicles remain, how-
ever, to be determined. While the role of estrogens remains unclear during follicular growth in primates [80], androgens sustain early follicular development in monkeys [81]. This effect of androgens, acting through their receptors present in follicles [82], can be due to their ability in stimulating GC proliferation of follicles at all stages of development [83], an effect that might be mediated by KL, BMP15, GDF9 and HGF [84]. It can therefore be assumed that in absence of FSH, local factors are able to support early follicle growth. However, many experiments have demonstrated that FSH acts in synergy with local factors to enhance follicle growth in laboratory rodents [69,70,85,86]. Finally, taken together, these observations lead to the conclusion that in conditions of FSH deprivation, the development of early growing follicles can be sustained by local factors. However, it appears likely that these factors are less efficient to sustain growth than they are when they act in synergy with FSH.

3.4.2. Follicles grow at a slow, but increasing, rate during basal follicular growth

In humans, despite the positive role played by FSH and local factors on their development, follicles smaller than 2 mm grow slowly [50]. Inhibitory factors produced by the follicle itself can counteract FSH- and local factor-induced GC mitotic activity. In rats, activin A, produced by large preantral – early antral follicles, can inhibit the FSH-induced growth of smaller follicles (100 – 120 μm) [87]. In the mouse ovary, AMH inhibits the FSH-stimulated follicle growth by repressing synthesis of some proteins involved in GC proliferation such as GDF9, BMP15, activin receptor 1, c-kit and TGF-β2 [88]. AMH inhibits the FSH-stimulated follicle growth by repressing synthesis of some proteins involved in GC proliferation such as GDF9, BMP15, activin receptor 1, c-kit and TGF-β2 [88]. These observations suggest that AMH, which have a similar pattern of expression in the primate ovary, may partly inhibit GC mitotic activity in humans. They may also explain why the growth rate of small follicles increases with their diameter [50], indeed, AMH being mainly produced in preantral and early antral follicles, progressively decreases as the follicle diameter increases [89]. In addition to the decreasing levels of AMH, the increasing levels of androgens produced by TICs might explain the increasing growth rate as follicles enlarge [81].

3.4.3. Granulosa cells are undifferentiated during basal follicular growth

Another characteristic of early growing follicles is that their GCs are undifferentiated, i.e. they express neither aromatase, enzymes for synthesis of progestins, nor LH receptors [63,90,91]. Whereas some local factors promote proliferation of GCs, they also inhibit their FSH-induced differentiation. EGF and FGF2 in the rat [92], and GDF9 and BMP15 in the mouse [93] inhibit FSH receptor mRNA expression, and subsequent GC differentiation during early follicular growth. In humans, TGF-α inhibits FSH-induced aromatase [94]. HGF suppressed the forskolin-induced StAR and progesterone synthesis by a steroidogenic GC tumor line [95] and KGF inhibited basal or hormone-stimulated progesterone production by GCs [74]. The high intrafollicular levels of certain IGFs decreases the bioavailability of IGFs, which stimulates both GC proliferation and differentiation and could be partly responsible for the slow growth rate of these follicles and their poor GC differentiation [96].

Taken together, these data show that the TICs, GCs, and the oocyte of early growing follicles produce factors that not only sustain their growth in the absence or in the presence of FSH, but also inhibit, at least partly, FSH-induced GC differentiation.

4. The selectable follicle and selection of the follicle destined to ovulate

Healthy follicles measuring 2 to 5 mm, referred to as selectable follicles, are observed at all stages of the menstrual cycle. During the late luteal phase, their number and quality rise in humans [97] as in monkeys [98] in response to increasing peripheral FSH levels following the demise of the cyclic corpus luteum. At this time, their number is between three and 11 per ovary in 24- to 33-year-old women [99], but it strongly decreases with ageing. It is among these follicles that the follicle destined to ovulate during the subsequent cycle will be selected [97,100]. Whereas early growing follicles are unresponsive to cyclic hormonal changes, selectable follicles are more receptive to these alterations. Their GC mitotic index strongly increases from the mid- to the late luteal phase [101] and they are highly responsive to exogenous gonadotropins in terms of GC proliferation [102]. The selectable follicle is a key step in folliculogenesis. As it develops very quickly, its metabolic requirements are high, leading to production of high levels of free radicals requiring action of detoxifying enzymes. Transgenic mouse deficient in γ-glutamyl transpeptidase, an enzyme involved in glutathione metabolism, exhibit a block in folliculogenesis at the mouse preantral stage, that corresponds to the selectable follicle in humans [103].

However, the intrafollicular concentration of estradiol in selectable follicles is very low when compared to that of androgens (Table 1). The follicular fluid of selectable follicles contains high levels of TGFα and EGF [78,104], as well as of IGFBPs [105] that inhibit FSH- [106] and IGFII- [107] induced aromatase, respectively. In addition, they also exhibit high levels of activin that decreases production of thecal androgens [108]. Nonetheless, the latter could be counteracted, at least partly, by an increasing number of LH receptors [109], an increasing LH pulse frequency during the early follicular phase [110] and the positive effect of GDF-9 on androgen production by TICs [111], leading to a significant activity of enzymes involved in androgen production in selectable follicles (Fig. 4B).

In conclusion, it appears that when follicles reach the selectable stage, their GCs become responsive to FSH in terms of proliferation, but not in terms of estrogen production.

Every mammalian species is characterized by a fixed number of follicle(s) that ovulate at each cycle (ovulatory quota). The term selection has been used by Goodman and Hodgson [112] to indicate the final adjustment of the cohort of growing follicles to this ovulatory quota. Each growing follicle possesses a FSH threshold requirement that should be surpassed to ensure ongoing preovulatory development [113]. It has been suggested that the selected follicle is the one which is most rapidly growing in response to the intercycle rise in FSH, i.e. the one with

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the lowest FSH threshold [97]. During the late luteal phase in the oldest ovulating women [114], or at the beginning of the follicular phase in younger women, the largest healthy follicle appears to be the selected follicle. It grows at a quicker rate than do other subordinate follicles [50], contains a detectable level of FSH [115] and differs substantially from selectable follicles by its estradiol concentration in the follicular fluid (Table 1). Taken together, these data strongly suggest that in the selected follicle, stimulators of both GC proliferation and differentiation are operating. Since IGFs promote both human GC proliferation and differentiation as well as production of androgens by theca cells, the importance of the IGF system in regulating FSH action has become increasingly apparent during the past decade [107]. Briefly, the IGF binding protein, IGFBP4, a potent inhibitor of FSH-induced steroid production by human GCs [116], is able to neutralize the IGF bioactivity, and especially that of IGF-II, the main IGF produced by human preovulatory GCs [105]. By stimulating an IGFBP-4 protease, the pregnancy-associated plasma protein-A (PAPP-A) in humans [117], FSH eliminates its inhibitory activity of IGFBP4 [118] and indirectly stimulates its own effect on both GC proliferation and steroidogenesis via IGF-II action. In conclusion, it can be proposed that the follicle with the lowest FSH threshold will be the first to exhibit an increased bioactivity of IGF-II, leading to an enhanced growth and differentiation of its GCs. Being the first to produce estradiol, to differentiate LH receptors on its GCs, to grow despite decreasing levels of FSH, to inhibit growth of less developed follicles, it will be the first to undergo preovulatory changes, then to ovulate. The view that the IGF system plays a key role during selection of preovulatory follicles was confirmed by the IGF-I KO mouse model in which folliculogenesis unfolds normally up to the selectable stage then is blocked thereafter [119].

5. Maturation of the preovulatory follicle

From the time it has been selected, the follicle destined to ovulate greatly enlarges from 6.9 ± 0.5 mm (2 to 5 × 10⁶ GCs) during the early follicular phase, to 18.8 ± 0.5 mm (50 to 100 × 10⁶ GCs) during the late follicular phase. So, during the early follicular phase, the selected follicle grows at a very high rate, requiring protection against free radicals. It is likely the reason for which transgenic mice deficient in superoxide dismutase 1, but not 2, have fewer large antral follicles than wild type mice [120]. During this final phase of growth, the GCs are subjected to marked morphological transformations resulting from a modulation of the actin cytoskeleton organization [121].

5.1. Preovulatory maturation before the mid-cycle gonadotropin surge

From the time it has been selected, the follicle destined to ovulate shows very marked changes in its steroidogenic activity. On the one side, production of androgens by the TICs is enhanced in response to increasing production of inhibin A [122,123] that strongly stimulates P450c17/17α/lyase (Fig. 4C). On the other side, aromatase, which is detected only in GCs from follicles ≥ 10 mm [90] (Fig. 4F), is stimulated by an increasing production of IGF-II [124], while NGF stimulates both FSH receptors synthesis and estradiol production [37]. Taken together, these changes lead to a maximal intrafollicular estradiol concentration at the time of the plasma estradiol peak. So, from the early to late follicular phase, estradiol in human follicular fluid increases from 658 to 2,583 ng/mL (Table 1). At the same time, intrafollicular concentrations of 17α-OH progesterone and progesterone increase (Table 1); these progestins are mainly produced by TICs, since 3β-HSD is only faintly present in GCs (Fig. 4D) [125]. As the follicle matures, the circulating levels of FSH decrease in response to estradiol and inhibit produced by the follicle itself, and GCs become able to bind LH [63,109]. Thus, it has been demonstrated that LH optimizes late folliculogenesis when administered with recombinant human FSH, by accelerating follicle growth and reducing both FSH dose requirements and development of small follicles [126]. This observation supports the view that when LH enters the follicle during the late follicular phase, it may replace FSH, as the principle regulator of GC differentiation.

From the mid-follicular phase, the primate preovulatory follicle becomes a highly vascularized structure [127]. The considerable increase in thecal blood vascularization occurs as a result of an active endothelial cell proliferation of thecal blood capillaries induced by angiogenic factors such as vascular endothelial growth factor [128].

5.2. Preovulatory maturation after the mid-cycle gonadotropin surge

After the mid-cycle gonadotropin surge, dramatic metabolic and morphologic changes occur simultaneously within the preovulatory follicle that switches from an estradiol-producing to

<table>
<thead>
<tr>
<th>Follicle type</th>
<th>E2 (pmol/L)</th>
<th>A + T + DHT (pmol/L)</th>
<th>17α-OHP (pmol/L)</th>
<th>P (pmol/L)</th>
<th>Total (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atretic (1–5 mm)</td>
<td>20 ± 5</td>
<td>794 ± 90</td>
<td>–</td>
<td>73 ± 13</td>
<td>887</td>
</tr>
<tr>
<td>Selectable</td>
<td>15 ± 5</td>
<td>638 ± 113</td>
<td>713 ± 318</td>
<td>130 ± 45</td>
<td>783</td>
</tr>
<tr>
<td>Newly selected</td>
<td>658 ± 38</td>
<td>487 ± 128</td>
<td>460 ± 112</td>
<td>440 ± 74</td>
<td>2712</td>
</tr>
<tr>
<td>Preovulatory 1</td>
<td>1270 ± 161</td>
<td>542 ± 176</td>
<td>1002 ± 212</td>
<td>1228 ± 228</td>
<td>4829</td>
</tr>
<tr>
<td>Preovulatory 2</td>
<td>2396 ± 348</td>
<td>203 ± 37</td>
<td>1812 ± 142</td>
<td>2464 ± 226</td>
<td>6146</td>
</tr>
<tr>
<td>Preovulatory 3</td>
<td>2583 ± 228</td>
<td>287 ± 44</td>
<td>2034 ± 326</td>
<td>7773 ± 643</td>
<td>10995</td>
</tr>
</tbody>
</table>
a progestin-producing structure. Progesterone receptors appear on GCs whose proliferation is arrested [129], capillaries from the theca layers invade the granulosa wall and both mural and cumulus GCs dissociate.

The production of steroids strongly increases, rising from a mean follicular fluid concentration of 4,800 ng/ml before the surge, to 11,000 ng/ml after (Table 1). The high levels of progesterone result from additive effects. As a result of the breakdown of the basal lamina, the cholesterol substrate required by GCs for progestin production, and which is provided to cells in the form of lipoprotein-bound cholesterol, can now reach the follicle via the blood supply [121]. After the gonadotropin surge, both 3ßHSD [91,125] (Fig. 4E) and P450scc [90] appear in GCs, where the levels of adrenodoxin and steroid acute regulatory protein (StAR) proteins, which allows entrance of cholesterol into mitochondria, are significantly increased. While the follicular progestin production strongly increases, that of androgens and estradiol dramatically drops.

Some hours before ovulation and in response to angiogenic factors produced by GCs [128], the granulosa wall, which was avascular before the mid-cycle gonadotropin surge, appears to be bloody after invasion of blood vessels originating from the theca.

LH causes a significant decline in gap junctions [121] leading to dissociation of mural GC and expansion of the cumulus-oocyte complex (COC) that is a highly specialized inflammatory-related process, which is obligatory for successful ovulation and involves activation of a variety of genes [130,131]. COC expansion requires action of local factors produced by GCs and the oocyte in response to a direct or indirect action of LH. Some of them can be mentioned: EGF family members such as amphiregulin, epiregulin, which are present in the human ovary [132], and β-cellulin, are produced by mural, but not cumulus GCs in response to LH and are mandatory for COC expansion and oocyte maturation [133]. Prostaglandin E (PGE) is also involved, since mice lacking the PGE-R2 do not ovulate because of defects in COC expansion [134]. The release by the oocyte of both BMP15, whose production may be upregulated by FSH [135], and GDF9, that stimulates, among others, expression of hyaluronan synthase and cyclo-oxygenase 2 [93], is associated with cumulus expansion.

After the LH surge, the oocyte resumes its meiosis and progresses from prophase 1 to metaphase 2 at the time of ovulation. Phenotyping of mice deficient for specific genes [131], and oocyte gene-expression profiles [136] have recently brought significant contributions to the knowledge of factors regulating both cytoplasmic and nuclear oocyte maturation.

6. Conclusion

There is no doubt that our knowledge of how follicles develop in the ovary has greatly increased during the last years. Whereas the role of FSH and LH as primary triggers of folliculogenesis remains indisputable, the role played by local factors has become increasingly apparent during the past decade. Both granulosa and theca cells, and more surprisingly, the oocyte, produce a variety of peptides that act broadly to influence gonadotropin action, either positively or negatively. In this respect, it is fascinating to note how large is the number of follicular functions that are controlled by a balance between activating and inhibiting factors. However, despite a better understanding of folliculogenesis in the rodent ovary, many processes remain poorly understood in humans, mainly because of the impossibility to make experimental studies. For example, depletion of the ovarian reserve, either by apoptosis or by activation of resting follicles, as well as acquisition by GCs of their fully responsiveness to FSH, are, among others, fascinating issues that constitute exciting goals to reach for improvement of women’s health and fertility in the future.

Conflicts of interest

None.

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