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Regulation of Ovarian Follicular Development in Primates: Facts and Hypotheses

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I. Introduction

IN THE adult ovary, folliculogenesis starts when follicles leave the pool of resting follicles (RF) to enter the growth phase. From there, the early growing follicle undergoes a developmental process including a dramatic course of cellular proliferation and differentiation. In primates, only one follicle commonly reaches the preovulatory stage every cycle; most follicles fail to complete this maturation scheme, dying in the process termed atresia. In recent years, a picture has emerged depicting the classic endocrine control of ovarian function by LH and FSH, entangled in a maze of regulatory systems hinging on cell-cell interactions between follicular cells, via action of a variety of molecules (1–3). Different types of cell-cell interactions have been described. In paracrine regulations, a molecule synthesized by one cellular type is released into the interstitial milieu to act directly on another cellular type. In autocrine regulations, molecules synthesized by one cellular type are released to act either on the same tissue (autocrine action), on immediate neighbor cells (juxtacrine action), or are not released and act inside the producing cell itself (intracrine action). These paracrine and autocrine regulations may be involved at every stage of folliculogenesis by acting either alone or by modifying the multiple functions of FSH and LH. Finally, it appears from these findings that during folliculogenesis a finely tuned process of progressive differentiation occurs in all constituents of the follicle.

Recent articles have described the anatomic, physiological, and endocrine changes that occur within the ovary during the female reproductive cycle (4–7). In addition, many *in vitro* experiments describing actions of various types of molecules on follicular cell function have provided new clues as to the regulations involved in folliculogenesis. Despite the new and pertinent insights recently obtained, data are sometimes confusing or contradictory, and the *in vitro* studies are limited. These limitations include: 1) sera used to improve culture conditions contain various factors that interact with the studied tissue, possibly leading to paradoxical or artifactual results; 2) the precise cell to cell interactions related to the structural organization of the follicle cannot be maintained; 3) any functional differences of individual cells within a cell type are obscured; and 4) because they are too small to be subjected to dissection and cell preparation procedures, resting, secondary, and preantral follicles are not easily investigated, especially in women with very low density due to both the size of the organ and the age of the subject. In addition, transposition of these data to primates *in vivo* must be performed with extreme caution since many of the findings are from various animal models with folliculogenesis patterns that are different from primates.

For these reasons, the mechanisms that control *in vivo* ovarian folliculogenesis in primates remain poorly understood. Since it is now possible to quantify certain biological activities and localize enzymes, hormones, and intraovarian peptides as well as their receptors in/on follicular cells, one of the approaches taken to acquire additional insight into the actual behavior of follicles is to evaluate *in situ* their functional status at various times during the ovarian cycle. Consequently, in an attempt to build a dynamic model of folliculogenesis, these functional data have been analyzed and integrated in the light of the well known cyclic endocrine changes and of more hypothetical intraovarian/extraovarian regulations occurring at each given stage of follicular development. Thus, the aim of this review is to use a chronological perspective to review follicular development in primates, beginning with the initiation of follicular growth and ending with preovulatory changes occurring in the dominant follicle. The putative control mechanisms that may operate *in vivo* to ensure the successful maturation of a single preovulatory follicle have been particularly emphasized. Most of the concepts presented derive from studies in humans and subhuman primates. When appropriate data were not available in primates, extrapolations were made from data obtained in rodents and farm animals.

II. The Stock of Resting Follicles

In the human ovary, follicles begin to form during the fourth month of fetal life (8). Although some of these newly formed follicles start to grow almost immediately, most of them remain in a resting stage until they either degenerate or some signal(s) activate(s) them to enter the growth phase. These follicles constitute the stock of RF. Three types of RF (primordial, intermediary, small primary) have been defined in humans (Fig. 1). The RF stock is mainly comprised of primordial and intermediary follicles, which are seen in similar numbers in the right and left ovaries in both humans (9) and monkeys (10).

At birth, the human ovary contains from 266,000 to 472,000 RF (11–13). With increasing age, the population of RF progressively decreases, leading to a stock at menopause estimated between less than 100 (13) and 1000 (14) RF. The age-related depletion of the RF stock occurs as a result of two processes: atresia and entry in growth phase. In the human ovary, the percentage of atretic RF, which has been estimated to be about 50% at birth (8), decreases from birth to approximately 30 yr of age (13). Up to 30 yr of age, the loss of RF is mainly due to RF atresia; thereafter, this loss of RF is due mainly to entrance of RF in growth phase. The sequence whereby the RF pool first decreases by atresia and then by entrance into the growth phase has been observed also in rodents during puberty and adult life, respectively (15, 16). The decay rate of RF accelerates from approximately 38 yr of age onward and is either due to an accelerated entry of RF in growth phase (13) or to an increasing atresia of RF (17). The discrepancy between these two studies can be explained by the follicular classification used by the authors, in which intermediary and small primary follicles were considered either as RF (13) or growing follicles (17).

A. Depletion of the stock by follicular atresia

Eosinophilia of the ooplasm, contraction followed by clumping of the chromatin material, and wrinkling of the oocyte nuclear membrane have been considered signs of RF atresia in human fetal and neonatal ovaries (8).

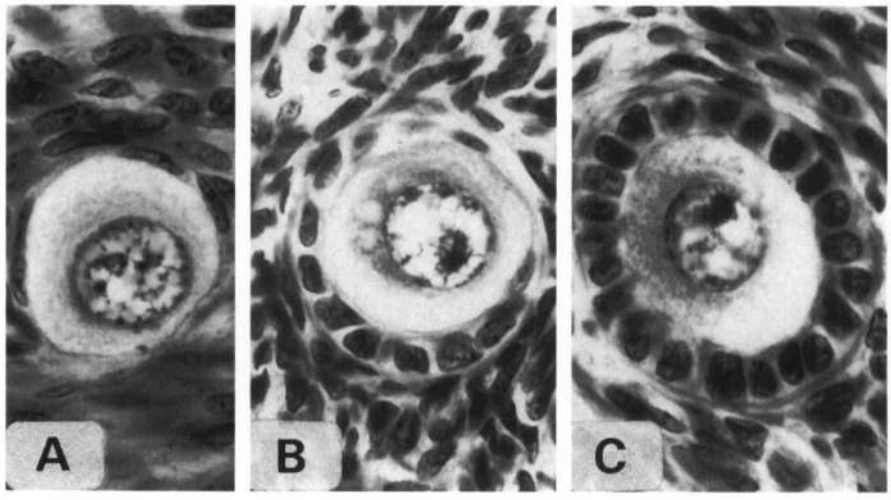

FIG. 1. Resting follicles in the adult human ovary, ×570. A, Primordial follicle in which the oocyte is surrounded by flattened GCs. B, Intermediary follicle in which the oocyte is surrounded by a mixture of flattened and cuboidal GCs. C, Primary follicle in which the oocyte is surrounded by a single layer of cuboidal GCs. [Reproduced with permission from A. Gougeon, *The Ovary*, pp. 21–39 (5).]

There are no data concerning the factors subserving atresia of RF in primates.

In postnatal normal rodents, plasma FSH is high at birth and between 7 and 18 days of life (18), and during the first 2 weeks of life the RF stock is halved (19). Using rodents showing natural (20, 21) or experimentally (22) low circulating levels of gonadotropins, it has been suggested that in prepubertal animals postnatal gonadotropins prompted follicular loss by atresia. Yet, nonphysiological levels of gonadotropins (23, 24), hemicastration (25), and ovarian transplantation in a gonadotropin-rich environment (26) have all failed to accelerate RF atresia. Alternatively, postnatal RF atresia may be the continuation of the massive oocyte attrition occurring in fetal ovaries (4). It has been recently suggested that expression of the protooncogene receptor c-*kit* is essential for germ cell survival (Ref. 27 and references therein). Therefore, a defect in the W locus that encodes this protein may have occurred during the multiple successive divisions of germ cells during fetal life and could be responsible for the observed massive neonatal RF atresia.

In adult rodents, it has been proposed that cyclic levels of gonadotropins may protect RF from atresia (21). However, in hypophysectomized mice and rats presenting undetectable levels of pituitary hormones, RF atresia is low (28, 29). Consequently, pituitary factors, including an unidentified atresia-promoting factor, have been speculated to play a direct or indirect role in RF atresia (28).

Hence, we must admit that the mechanisms regulating the RF depletion by atresia in both juvenile and adult animals are not clearly identified and require further studies.

B. Depletion of the stock by entry of follicles in growth phase: initiation of follicular growth

1. Morphometric studies. Follicular growth may begin at any time of the female's reproductive life, as early as infancy, and continues throughout puberty, during the ovarian cycle, and pregnancy, until the end of the reproductive period (15). The precise time at which follicular growth actually starts is difficult to establish.

In humans, primordial, intermediary, and small primary follicles differ in their diameter, due to differences in the number and size of granulosa cells (GCs), but they do not differ in mean diameter of their oocyte and its nucleus (9). In addition, studies performed in rats have indicated an extremely slow proliferation rate of GCs from follicles containing between five and eight GCs in the largest cross-section (30). This illustrates the very slow process by which primordial follicles are transformed into small primary follicles, which is seen more as a maturation than a growing process.

In humans, evolution of morphometric parameters of RF and early growing follicles shows that active follicular growth starts when the oocyte nucleus reaches a critical diameter of 19 μm and when approximately 15 GCs are present in the largest cross-section (9). This finding is in keeping with observations made in mice and rats where the entry into the growth phase takes place when eight to 20 GCs are present in the largest cross-section (31, 32). In rodents, nucleoplasmic RNA syntheses may be the first event in the initiation of oocyte growth in response to an inductive stimulus coming from surrounding follicular cells (31, 33) or from the oocyte itself (32).

2. Factors triggering the initiation of follicular growth. The possible existence of a factor triggering the initiation of follicular growth remains an intriguing and unanswered question.

a. Is there an inhibitory factor preventing RF from leaving the stock? In humans (13) as in other mammals, a strong correlation exists between the RF stock size and the number of growing follicles. The larger the stock, as in the infant ovary, the greater the number of follicles beginning to grow. With aging, as follicles continuously leave the RF stock, the number of growing follicles decreases, but the proportion of primary and early growing follicles increases in primates (9, 34) as in mice (35). This increase may be triggered by the progressive disappearance of an inhibitory influence on primary follicle growth exerted by primordial and intermediary follicles themselves, acting via an unknown mechanism whose magnitude is directly proportional to the size of the stock. This putative mechanism may represent an adaptive process, allowing the ovary to promote the growth of a sufficient number of follicles to permit one of them to achieve its complete development and ultimately to ovulate, despite declining numbers of available follicles. However, the size of the RF stock does not appear to be the only factor controlling

the number of follicles starting to grow per time unit, since, during pregnancy in mice, this number decreases (36).

b. Is there a stimulus acting on the RF store that stimulates some follicles to leave it? Data concerning a possible stimulatory role for gonadotropins in the initiation of growth phase in immature primates appear confusing. In contrast to the hypophysectomized monkey fetus in which initiation of follicular growth does not appear blocked (37), there are no early growing follicles in the anencephalic human fetus (38).

Despite some studies suggesting that gonadotropins are involved in the initiation of follicular growth in immature rodents (24, 39, 40), they have been confirmed neither by observations of ovaries coming from mice bearing a natural deficiency of gonadotropins (20, 21) nor by transplantation of neonatal rat ovaries into recipient adult females exhibiting either high or low levels of circulating gonadotropins (26). In addition, the efficiency of gonadotropins in initiating RF growth between days 1 and 5 of life is in apparent contradiction with recent data on presence of gonadotropin receptors in RF. Although shortened receptors for FSH (FSH-R) can mediate a response to FSH (41), full-length transcripts for the FSH-R have not been detected in the mouse ovary until day 5 of life (42). FSH-R and LH receptor (LH-R) only appear in the ovary from days 5 to 11 (43), whereas immunostaining for FSH and LH to rat RF was shown only from day 8 of age (44). Thus, data from immature rodents suggest that initiation of follicular growth may be independent of a gonadotropic effect.

During adult life in rodents, initiation of follicular growth may be controlled by gonadotropins (20, 21, 45). In humans, the acceleration in the decay rate of RF reported after 38 yr of age (13) may be linked to the significant increase in circulating FSH levels in women over 35 yr of age (46). Nevertheless, during natural hypopituitary conditions in both animal species and humans, the initiation process is not completely abolished (20, 21, 47). Two studies in mice (24) and rats (26) and more recent findings in monkeys, in which a GnRH agonist (GnRH-a) blocked the transition between intermediary and primary follicles (34), suggest that gonadotropins may act on the transformation of flat GCs into cuboidal GCs. Rather than initiating follicular growth, *per se*, gonadotropins may act on RF maturation by transforming their GCs, making it possible for these follicles to enter the growth phase in response to an unknown signal.

Recent findings indicate that some of the genes coding for certain proteins, growth factors and their receptors (48–53), or protooncogenes (27), which are present in GCs and/or the oocyte of small follicles, may be involved in the transition of RF to growing follicles. For example, it has been suggested that pituitary hormones such as TSH may act synergistically with FSH to enhance the entry of RF in the growth phase (20). Thus, the *erbA* oncogene, which encodes a thyroid hormone receptor, is expressed more abundantly in GCs from small follicles than in GCs from large follicles (54). It has also been proposed that expression of *steel*, encoding the Kit ligand and present in GCs, may be involved in stimulation of oocyte growth via c-*kit* receptor tyrosine kinase present on this cell (27), and that abundant expression of myc protein in the human primordial oocyte may play a role in the initial growth of the oocyte (55). Finally, in primordial and preantral follicles, the retinoblastoma gene product (pRb) expression is different in GCs and in the oocyte (33). In primordial follicles, the nonproliferating flat GCs show low pRb expression, but the oocyte exhibits strong nucleolar pRb staining. It has been suggested that pRb expression in the oocyte nucleolus is associated with production of negative growth factors that may inhibit proliferation of GCs. During resumption of follicular growth, GCs show increased pRb staining, and the enlarging oocyte shows pRb depletion. These data suggest that the resting state of the primordial follicle may be maintained by the oocyte, and resumption of follicular growth may be initiated by GCs. To overcome negative signaling from the resting oocyte, the GCs of human primordial follicles may require stimulation by factors arising from vascular pericytes such as the Thy-1 differentiation protein (33).

Although the number of follicles entering growth can be modulated either by the size of the pool or by endocrine factors, recent studies suggest multiple paracrine/autocrine factors acting in an elusive fashion. In conclusion, we must admit that mechanisms triggering atresia of RF and initiation of follicular growth remain largely unknown, especially in primates. Consequently, identification of the chemical nature and cellular source of the putative inducer of follicular growth initiation remains a promising field of investigation.

III. Morphological Aspects of Follicular Development

In this section, the anatomical and dynamic aspects of the primate folliculogenesis will be described from the entry of a follicle into the growth phase until it becomes preovulatory.

A. Description of the follicular development in primates

When follicles enter the growth phase, they enlarge, both by proliferation of GCs and by an increase in size of the oocyte. A zona pellucida begins to be laid down around the oocyte shortly after initiation of follicular growth; however, its exact cellular source remains unclear (56). Progressively, follicles become secondary follicles (Fig. 2A). At this stage of development some stroma cells near the basal lamina become aligned parallel to each another. As the follicle enlarges, this surrounding connective tissue stratifies and differentiates into 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 (TI), some fibroblast-like precursor cells change into polyhedral cells, also referred to as epithelioid cells (Fig. 2B). Morphological studies have shown that definitive theca layers appear only when follicles contain three layers of GCs (follicle diameter: 100–125 μm) in monkeys (M. J. Koering, unpublished data), and three to six layers of GCs (follicle diameter: 103–163 μm) in women (A. Gougeon, unpublished data). The smallest early growing follicles lack an independent blood supply, but secondary follicles 80–100 μm in diameter are served by one or two arterioles, terminating in an anastomotic network just outside the basal lamina (57). The physiological importance of this event is emphasized by the fact that the follicle becomes directly exposed to factors circulating in the blood.

From the time of appearance of epithelioid cells in the TI, the secondary follicle is defined as a preantral follicle (Fig. 2C) and constitutes the first class of growing follicles in a

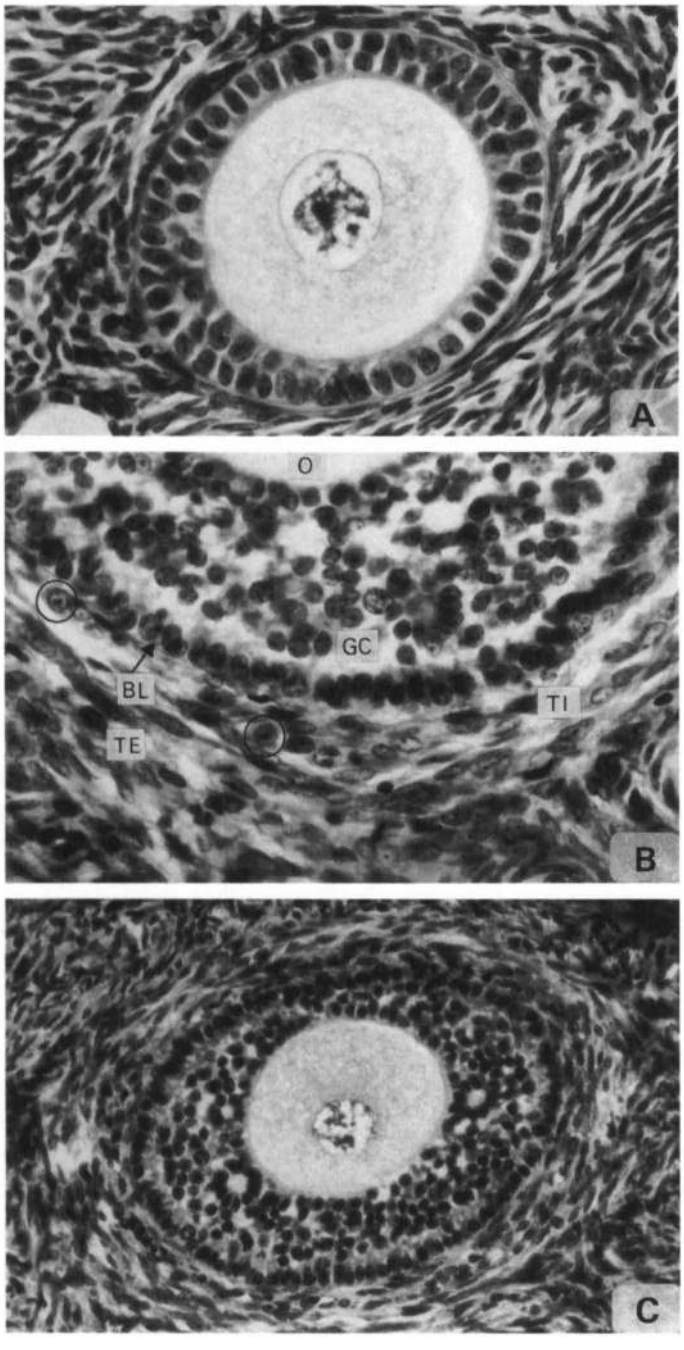

FIG. 2. Human ovarian follicles with undifferentiated GCs. A, Secondary follicle, ×275. B, High power micrograph of epithelioid TICs (into *open circles*) from a preantral follicle, ×480. C, Preantral follicle (class 1), ×350. GC, Granulosa cell; TI, theca interna; TE, theca externa; BL, basal lamina. [A-B reproduced with permission from A. Gougeon, *The Ovary*, pp. 21–39 (5).]

classification based on morphological aspect and total number of GCs in each individual follicle (Fig. 3).

Appearance of an antral cavity starts with the development of small fluid-filled cavities of 40 μm in diameter that aggregate to form the antrum (Fig. 4A). From this point, the GCs surrounding the oocyte constitute the "cumulus oophorus," In primates, as in most mammals, shortly after the follicle acquires a single antral cavity, the GCs that border the basement membrane lose their cuboidal shape and assume a columnar appearance, while the remaining GCs remain cuboidal (4). In ewes, these columnar GCs exhibit a greater rate of cell proliferation than do GCs in the antral region (58). In humans and monkeys, follicles pass from the preantral (class 1) to the early antral stage (class 2) at a follicular diameter between 180 and 250 μm (10, 59). Through the accumulation of fluid in the antral cavity and proliferation of GCs and TI cells (TICs), the follicle progresses through subsequent stages of development (Fig. 4B, class 3; and Fig. 4C, class 4) until it reaches a size of about 2 mm.

Healthy follicles measuring 2–5 mm (class 5), referred to as selectable follicles, are observed at all stages of the cycle. Those that are present during the late luteal phase constitute the population from which the follicle destined for ovulation during the subsequent cycle will be selected (60, 61). Their number in the late luteal phase is between three and 11 per ovary in 24- to 33-yr-old women (62) and strongly decreases with increasing age. In macaca monkeys, follicles of about 1 mm constitute the population of selectable follicles (63).

There is no healthy 5- to 9-mm follicle (class 6) during the midluteal phase, and they are rarely observed during the late follicular and luteal phases. During the early follicular phase, the newly selected follicle belongs to this class, and its diameter is between 5.5 and 8.2 mm (60). The size of the follicle destined for ovulation increases greatly during the follicular phase by cellular multiplication and accumulation of fluid in the antrum until the ovulatory gonadotropin surge, and then only by the latter process until ovulation. In humans, the diameter of the preovulatory follicle increases from 6.9 ± 0.5 mm (early follicular phase; range, 5.5–8.2 mm) to 13.3 ± 1.2 mm (midfollicular phase; range, 7.8–15.6 mm) and then to 18.8 ± 0.5 mm (late follicular phase; range, 15.2–26.9 mm) (60, 62). The mean number of GCs increases from approximately 2–5 million during the early follicular phase to 50–100 million at the time of ovulation (59). During the final phase of development, the granulosa layer of the follicle destined to ovulate is subjected to marked morphological transformations (Figs. 5 and 6) resulting from a modulation of the actin cytoskeleton organization, which may be intrinsic in the programmed events that lead to GC differentiation (64). While mitotic figures have disappeared after the midcycle gonadotropin surge, as many as 1.2% and 0.3% pyknotic GCs can be observed in monkey (65) and human (66) preovulatory follicles, respectively. Color Doppler studies showed that there is a direct correlation between the development of human ovarian follicles and their blood flow (67). Therefore, from the midfollicular phase, the primate preovulatory follicle becomes a highly vascularized structure with the area of the TI occupied by blood vessels being 2 times larger than in other follicles within the same or contralateral ovary (68, 69). This may occur as a result of an active endothelial cell proliferation in the TI throughout the follicular phase (70). Some hours before ovulation, the granulosa wall, which was avascular before the midcycle gonadotropin surge, appears to be bloody after invasion of blood vessels originating from the TI and occurring after the surge (70).

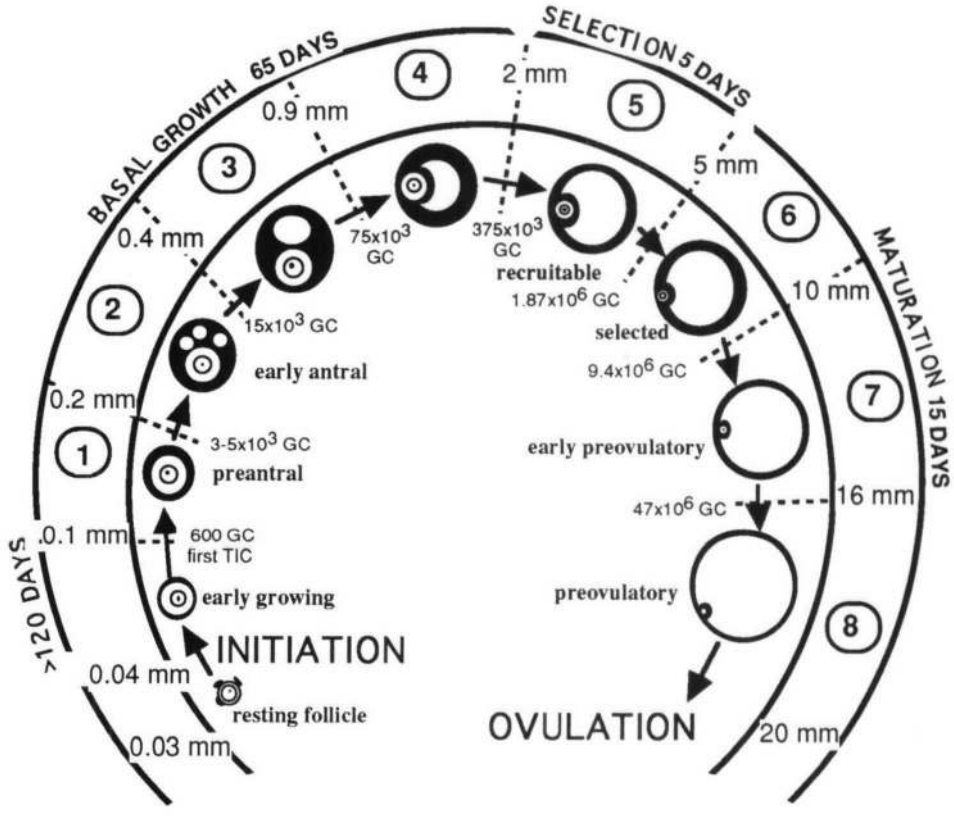

FIG. 3. Classification of follicles in the human ovary.

B. Cytological and morphometric changes of follicular tissues during development

During follicular development, the granulosa layer undergoes numerous changes (Table 1). Independently of its cyclic variations, the rate of cell proliferation increases as the follicle develops (71) and culminates in selectable follicles observed during the late luteal phase and in the newly selected follicle. The mean number of GCs per cubic millimeter of tissue decreases significantly as follicles mature. This corresponds to an increase in volume of individual GCs and can be considered as morphological evidence of GC maturation (66).

Ultrastructural studies show that immature GCs contain mainly rough endoplasmic reticulum, which reflects the preponderance of protein synthesis for growth and proliferation. A gradual increase in the extent of smooth endoplasmic reticulum and appearance of mitochondria with tubular cristae and lipid droplets occur as steroidogenesis increases in GCs from preovulatory follicles and culminate with maximal progesterone (P) secretion in the corpus luteum (64).

GCs communicate between themselves via two types of specialized cell contact systems. Gap junctions are aggregates of transmembrane channels, each composed of six identical subunits, or connexins, which are arranged to form a central pore (72). Adherent junctions are associations of filamentous dense material on the intercellular sides of the plasma membrane. Adherent junctions are present as early as the first stages of follicular growth whereas gap junctions appear during antral cavity formation and grow rapidly during expansion of the antrum. In GCs from human preovulatory follicles, approximately 30% of the entire plasma membrane is occupied by gap junctions. Whereas FSH increases acquisition of gap junctions by GCs, the gonadotropin surge or human chorionic gonadotropin (hCG) administration to preovulatory GCs causes a significant decline in gap junctions (64).

There are few data concerning morphological changes of TICs during follicular growth. Through mitosis of theca stem cells, their number increases, but to a lesser extent than do GCs.

C. Dynamics of human follicular growth

In the human, the time for early growing follicles to attain the preantral stage is not known. From data obtained in monkeys (73), where less than 10% of follicles possessing one to two layers of GCs contain GCs labeled with [^{3}H]thymidine, and rats (30), where the growth rate of small follicles is very slow, it can be hypothesized that this time is probably more than several months in humans.

Throughout development, the number of GCs increases in each individual follicle. Consequently, each follicle enters a given class when its number of GCs attains the lower limit of this class (characterized by a fixed number of GCs: Fig. 3). After a time lag (the transit time) directly proportional to its GC mitotic index, the follicle leaves this class when its number of GCs attains the upper limit of the class and enters into the subsequent class. Taking into account that from the preantral stage (class 1), the ovulatory follicle will pass through eight classes to reach the preovulatory size (class 8), the time required for this follicle to pass from the preantral to the ovulatory stage will be the sum of the transit times required

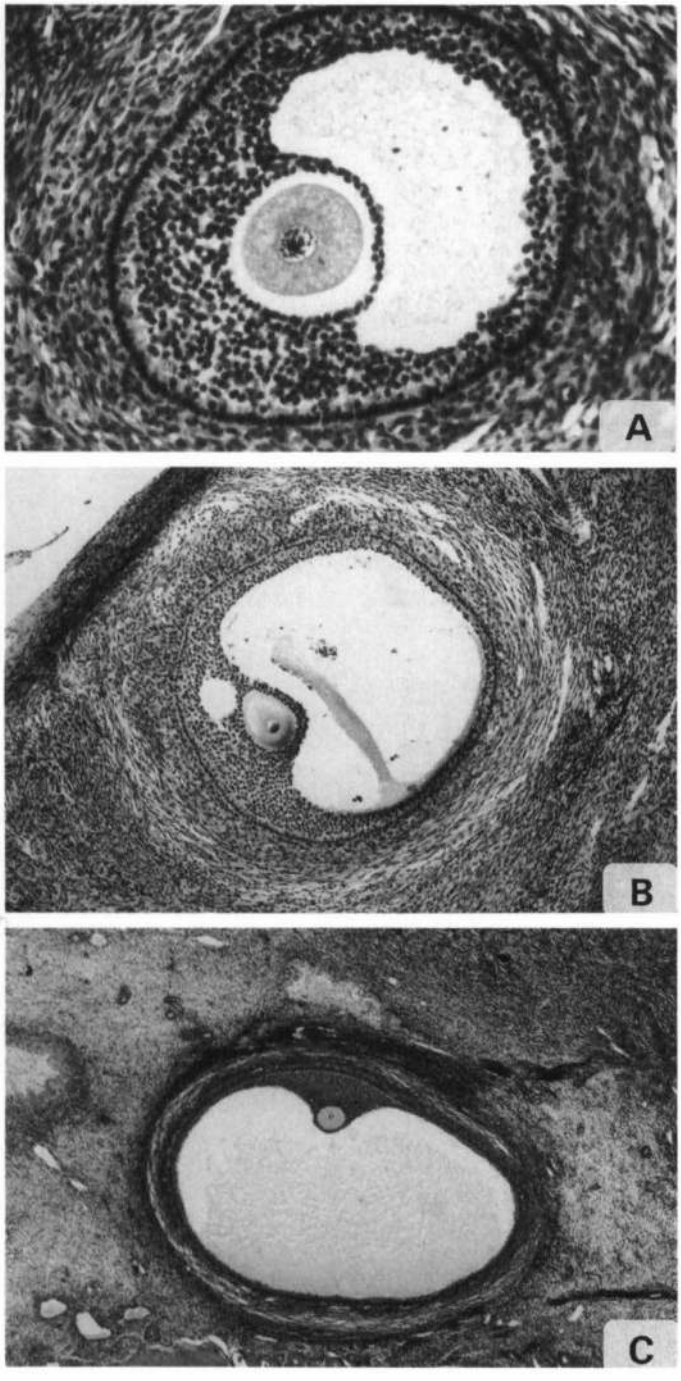

FIG. 4. Human ovarian follicles with undifferentiated GCs. A, Early antral follicle (class 2), ×170. B, Small antral follicle (class 3), ×60. C, Small antral follicle (class 4), ×25. [A and C reproduced with permission from A. Gougeon, *The Ovary*, pp. 21–39 (5).]

for this follicle to pass each class. These transit times have been calculated by two different techniques. The first measured the time interval separating the visible entry of follicles into one class (the number of healthy follicles increases and their mean size decreases, signifying that small follicles have entered the class) from the visible departure (when the population of follicles decreases) of these follicles to the subsequent class (71). These changes have a physiological significance when a departure of follicles from class x occurs simultaneously with an entry of follicles into class $x + 1$. Visible passages of follicles from one class to the next were observed during the late follicular phase when class 1 follicles enter class 2, between the late luteal and early follicular phases when class 2 follicles enter class 3, during the late follicular phase when class 3 follicles enter class 4, and during the late luteal phase when class 4 follicles enter class 5 (71). The second technique calculated the GC doubling time after *in vitro* determination of the duration of mitosis and estimated the mean mitotic index for each follicular type (Table 2). The duration of mitosis obtained, 1.42 h (71), was similar to that obtained in the ewe (74).

From these transit time values, going back from the preovulatory follicle to preantral follicles has suggested the dynamics of human follicular development (Fig. 7). Functional experiments studying the effects of follicle-ectomy or luteectomy, both in monkeys (75) and humans (76, 77), are in agreement with the dynamics of follicular growth, at least from the selectable stage onward.

IV. Gonadotropins and Follicular Functions

A. The primary role of gonadotropins to initiate and sustain follicular functions

The basic function of the ovulatory follicle is to produce a fertilizable oocyte and to function as an endocrine gland during its maturation and after ovulation through transformation into a functional corpus luteum. Preovulatory follicular cells, and especially GCs, become able to assume their full endocrine functions at the completion of a highly regulated process that leads them from a proliferative to a differentiated status. This process is under the primary control of two pituitary hormones, FSH and LH.

1. Gonadotropins sustain follicular growth. Whereas the role of gonadotropins in initiating follicular growth remains unclear, many studies have demonstrated their tropic action on follicle cells. In rodents, development of GCs is impaired in primary and secondary follicles when levels of circulating gonadotropins are drastically reduced either naturally or experimentally (21, 26, 78). In these situations, GC nuclei are irregularly shaped rather than round, the spaces between the GCs disappear, and GC proliferation is strongly impaired but not entirely inhibited. In all cases, injection of FSH restores the regular round shape of GC and development of small follicles (78, 79).

That gonadotropins and especially FSH are of primary importance to sustain follicular growth has been extensively reviewed (4, 6). Several examples illustrate this primary role of gonadotropins in humans. Various physiological and pathological situations are characterized by an unfavorable hormonal environment either due to low levels of circulating gonadotropins or to an unfavorable FSH/LH ratio, associated with anovulation. Women suffering from Kallman's syndrome (80) can be induced to ovulate with exogenous gonadotropins, and those suffering from a polycystic ovarian syndrome (PCOS) in which circulating levels of FSH are very low, can be induced to ovulate by treatment with high doses of FSH (81, 82). In normal women undergoing *in vitro* fer-

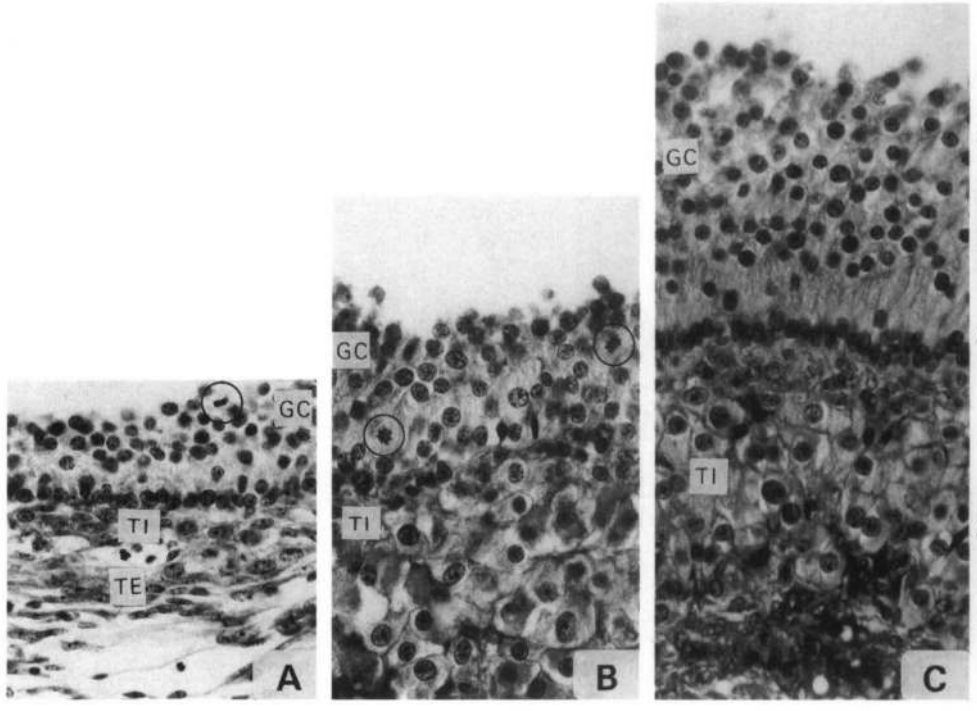

FIG. 5. Follicular wall changes in the follicle selected for ovulation (× 480) during the early (A) and midfollicular phase (B). The granulosa layer is more or less homogeneous, with some GCs that border the basal lamina assuming a columnar appearance, and polyhedral GCs exhibit a high proliferative activity. C, During the plasma E_2 peak, all the GCs that border the basal lamina assume a columnar appearance with nuclei ranked along the basal lamina, whereas other GCs remain polyhedral in shape with a declining mitotic activity. GC, Granulosa cell; TI, theca interna; TE, theca externa; mitosis are in *open circles*.

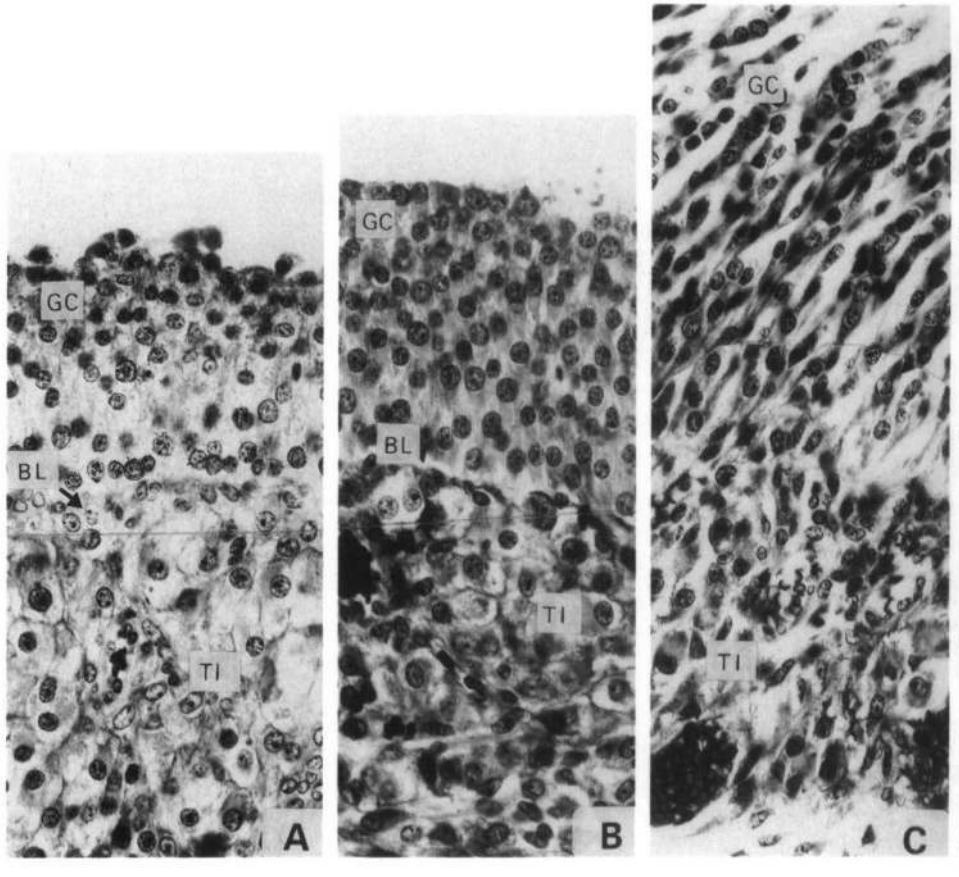

FIG. 6. Follicular wall changes in the preovulatory follicle from the midcycle gonadotropin surge (×480). A, When the preovulatory gonadotropin surge begins, the columnar appearance of the basal layer of GCs disappears, the basal lamina becomes blurred, and mitoses become scarce; the size of TICs is maximum. B, Just after the midcycle gonadotropin surge, the basal lamina is disappearing and GCs are homogeneously distributed, there are no more mitosis, and the size of TICs has decreased. C, A few hours before ovulation there are no more mitoses, GCs are dissociated, spindle-shaped, and radially arranged, and the granulosa wall appears to be bloody after invasion of blood vessels coming from the TI. GC, Granulosa cell; TI, theca interna; BL, basal lamina.

tilization and embryo transfer (IVF&ET), treatment with exogenous gonadotropins overrides the normal ovulatory quota by sustaining preovulatory growth of a large number of follicles (83). Moreover, recent data suggest an exclusive role for FSH in sustaining follicular development. In monkeys treated either with high doses of GnRH antagonist (84) or with a LH antiserum (85), leading to LH-deficient animals, FSH alone is capable of stimulating ovarian follicular growth.

2. *Gonadotropins sustain follicular steroidogenesis.* According to the "two-cell, two gonadotropins" theory (86), TICs are stimulated by LH to produce aromatizable androgens that are

TABLE 1. Morphometric characteristics of GCs in healthy growing follicles (numbers in parentheses) during folliculogenesis

Class (μm)	Number of GC	Mitotic index (%)	GC ($\times 10^{-5}$) per mm^3 of tissue	Thickness of GC layer
1 (742)	2,100 ± 0,100	0.384 ± 0.008	12.13 ± 0.17	[c]
2 (565)	7,600 ± 0,100	0.440 ± 0.008	11.08 ± 0.08	35.9 ± 1.0
3 (342)	33,300 ± 1,300	0.532 ± 0.011	10.45 ± 0.12	36.0 ± 0.5
4 (143)	176,800 ± 7,500	0.875 ± 0.031	9.82 ± 0.19	27.6 ± 0.8
5 (133)	933,500 ± 44,900	0.981 ± 0.040	8.53 ± 0.27	27.1 ± 0.9
6 (31)	3,400,000 ± 300,000	1.191 ± 0.125	7.46 ± 0.23	77.5 ± 4.2
7 (16)	24,140,000 ± 2,970,000	0.930 ± 0.170	6.29 ± 0.25	81.2 ± 3.2
8 (22)[a]	54,760,000 ± 4,610,000	0.639 ± 0.070	6.24 ± 0.13	97.8 ± 3.3
8 (16)[b]	59,070,000 ± 5,510,000	0.027 ± 0.007	4.59 ± 0.20	99.1 ± 3.2

Values are expressed as mean ± SEM.
[a] Before the midcycle gonadotropin surge.
[b] After the midcycle gonadotropin surge.
[c] Measurement not possible because of the absence of an antral cavity.

TABLE 2. Transit time for each follicle class; comparison of results obtained from *in vitro* studies and analysis of morphometrical parameter changes

Follicle class	Mitotic index (%)	Doubling time (h)	Transit time (days)	
			From doubling time value	From time interval between entry and exit
1	0.38 ± 0.01	246 ± 12	23.8 ± 1.2	25
2	0.44 ± 0.01	216 ± 5	20.9 ± 0.5	20
3	0.53 ± 0.02	171 ± 8	16.6 ± 0.8	15
4	0.87 ± 0.09	111 ± 6	10.8 ± 0.6	10
5	1.65 ± 0.18[a]	60 ± 5	5.8 ± 0.5	5
6	1.51<<2.01[b]	47.5<<62.9	4.6<<6.1	5
7	0.82<<2.38[c]	40.2<<115.6	3.9<<11.2	5

Values are expressed as mean ± SEM.
[a] During the late luteal phase.
[b] Range of individual GC mitotic indices observed during the early follicular phase.
[c] Range of individual GC mitotic indices observed during the midfollicular phase.

transported to GCs where they are converted to estrogens by aromatizing enzymes, which are induced by FSH. The human thecal steroidogenesis and its control by LH have been extensively documented (84, 87, 88), and the recent use of recombinant LH has confirmed previous observations (89). Briefly, the TI appears to be the primary site of androstenedione (A) synthesis, which is the principal aromatizable steroid, whereas testosterone (T) is produced in lesser amounts (90). The androgen secretion by TICs under LH stimulation appears to result from activity of enzymes, such as cholesterol side chain cleavage ($P450_{scc}$), 17α-hydroxylase/lyase ($P450_{17\alpha/lyase}$), and 3β-hydroxysteroid dehydrogenase (3βHSD).

The steroidogenic pathways in GCs are organized principally for the metabolism of androgens to estrogens before the midcycle gonadotropin surge and for *de novo* synthesis of P and its metabolites after the midcycle LH surge (91). Briefly, 17β-hydroxysteroid dehydrogenase (17HSD), which is present in GCs as soon as the primary stage (92) transforms A into T, and aromatase (also referred as $P450_{arom}$) transforms T into E_2 (88). Although aromatase has been immunohistochemically detected in TICs of follicles from the preantral stage onward (93), it has been estimated that during the preovulatory maturation, 99% of the aromatase activity resides in the GCs, which have at least 700 times the aromatase activity of the TI (94). The role of FSH in the induction of GC aromatase activity has been demonstrated in rats *in vivo* by using recombinant human FSH (95). During the late preovulatory maturation, acquisition of LH receptivity transforms the ability of GCs to respond to gonadotropins since aromatase can be stimulated by hCG (96). After the midcycle gonadotropin surge, 3βHSD is expressed, and GCs turn from a predominantly estrogen-producing to a progestin-producing tissue (66).

Data reported above indicate that gonadotropins have long been recognized as basic supports for follicular growth and steroidogenesis. However, *in vivo* analyses of folliculogenesis show that small and large follicles do not respond in a similar way to the same gonadotropic signal.

B. Changes in follicular responsiveness to gonadotropins during folliculogenesis

1. The basal follicular growth.

a. Morphological evidence supporting the existence of a basal follicular growth. Throughout human infancy, when the circulating levels of gonadotropins are lower than those recorded during puberty and adulthood, 2- to 6-mm follicles are observed in the ovaries, and some of them can exceed 6 mm (97) when circulating gonadotropin levels increase after 6 yr of age (98). During pregnancy, when plasma FSH levels are low or undetectable (99), the human ovary contains many small healthy and atretic antral follicles with diameters up to 6 mm (100). In patients suffering from Kallman's syndrome, levels of gonadotropins are very low (101). In these women, follicular development is markedly impaired (102); nevertheless, the observation of atretic antral follicles (47) and the

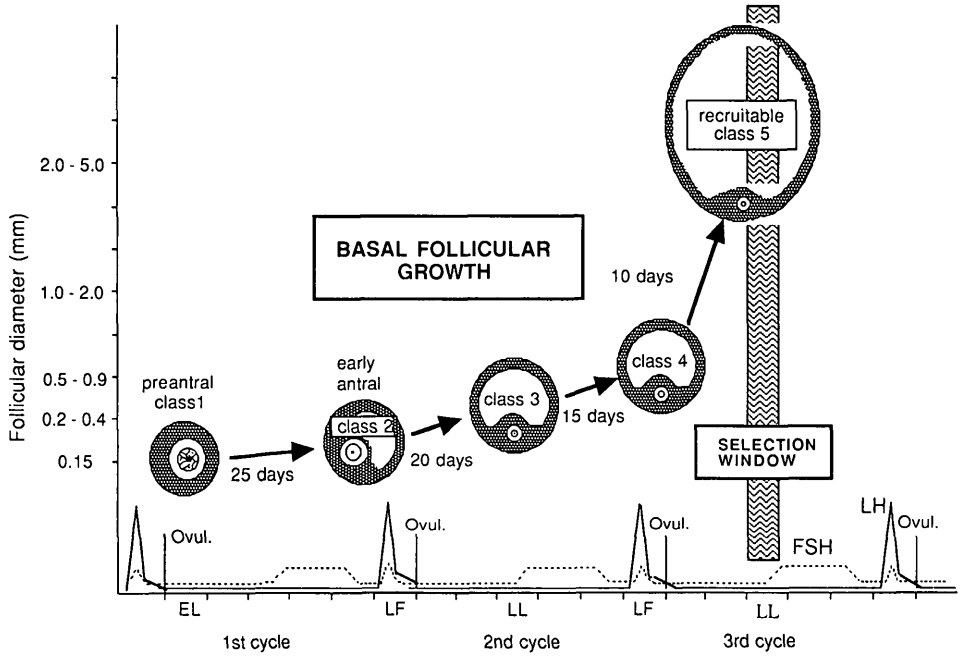

FIG. 7. Dynamics of the development of follicles belonging to the cohort from which the follicle destined to ovulate will be selected. Growth commences with the entry of follicles into class 1 during the early luteal phase (EL). Twenty-five days later, during the late follicular phase (LF) of the following cycle, an entry of follicles into class 2 can be seen. The end of the follicular phase is a favorable period for the appearance of the antrum since the circulating levels of FSH have been high during the preceding days, and this hormone promotes the initiation of antrum formation. Twenty days later, follicles pass into class 3 during the late luteal phase (LL) and 15 days later into class 4 during the late follicular phase (LF) of the subsequent cycle. Follicles enter into class 5 (selectable stage) 10 days later, in the late luteal phase (LL), and constitute a population from which the follicle destined to ovulate during the subsequent cycle will be selected. [Adapted with permission from A. Gougeon, *The Ovary*, pp. 21–39 (5).]

occurrence of ovulation within approximately 2 weeks after exogenous gonadotropin treatment (80, 82) suggest the presence of selectable follicles at the onset of treatment. In oligomenorrheic women, and in patients with polycystic ovaries showing a very low FSH/LH ratio (103), follicles can attain more than 5 mm. In the so-called "resistant ovary syndrome," women presenting with primary amenorrhea are usually hypoestrogenic despite high serum levels of FSH and LH. Ovarian biopsies from these women show a progression of follicular growth up to 1–2 mm (104).

Taken together, these findings indicate that in an inadequate gonadotropic environment, human folliculogenesis is impaired since ovulation does not take place. However, although the number of growing follicles in these conditions is more or less markedly reduced, follicular growth is not completely abolished since in the less favorable conditions, some follicles can grow to a diameter of approximately 2–5 mm, *i.e.*, the selectable stage. So, according to some authors (103), the growth of follicles from the preantral to the selectable stage only requires tonic (or basal) levels of gonadotropins. This could explain why healthy selectable follicles 2–5 mm in diameter are always present from infancy to menopause in humans, even when plasma levels of gonadotropins are low.

b. Low follicular responsiveness to gonadotropins during basal growth. Although the rate of GC proliferation increases as follicles enlarge (Table 1), it is low and not related to cyclic hormonal changes for follicles smaller than approximately 2 mm (classes 1–4) (105). In addition, these follicles seem to be unresponsive to exogenous gonadotropins. In women undergoing IVF&ET, the mean number of recovered oocytes after treatment with human menopausal gonadotropins (hMG) is grossly similar (106) to the number of selectable follicles (>2 mm) that are present during both late luteal and early follicular phases of the spontaneous cycle (60, 62, 107).

During basal growth, primate follicles exhibit slight steroidogenic activity. Small and large human preantral follicles cultured *in vitro* spontaneously produce very low amounts of P and A whereas E_2 production is not detectable (108). Cultured GCs from marmoset follicles 0.5–1 mm (109) and from human follicles smaller than 2 mm (110) possess only faint aromatizing capacity. In human growing follicles, immunolocalization of steroidogenic enzymes shows that neither $P450_{17\alpha}$ nor $P450_{scc}$ are present in TI from follicles smaller than 2 mm (111, 112). Immunoreactive 3βHSD has been detected in both TICs and GCs of preantral follicles (92); however, the staining is slight and possibly not significant. This observation is in contradiction with previous immunohistochemical studies showing that 3βHSD is restricted to TICs in follicles larger than 2 mm (113). Similarly, the presence of $P450_{arom}$ in follicles during basal growth is disputed. This enzyme has been reported to be either absent (112) or present (93) in GCs of these follicles, despite the same aromatase monoclonal antibody being used. These discordant results may be due to differences in the processing of the samples [either fixed paraffin section (93) or unfixed frozen sections (112)].

It can therefore be assumed that during basal growth, the development of human follicles requires only small levels of gonadotropins. These follicles are faintly responsive to cyclic

gonadotropin changes, and their GCs exhibit low proliferative activity, weakly stimulated by gonadotropins. In addition, except for 17HSD (92), steroidogenic enzymes are weakly or not expressed.

2. *The selectable follicles.* From a size of 2 mm, follicles become more dependent on FSH since their percentage of atresia decreases when FSH increases (105). From the mid- to the late luteal phase, granulosa of selectable follicles exhibit a significant increase in the rate of cell proliferation (105), paralleling the increase in circulating levels of FSH. In addition, when stimulated with hMG during both the late luteal and early follicular phase, selectable follicles can grow more quickly than unstimulated selectable follicles (114).

Observations made at various times during the menstrual cycle indicate that the intrafollicular steroid milieu from healthy and atretic 1- to 5-mm follicles is similar (Table 3). These follicles have a low intrafollicular estrogen-androgen ratio and A is the dominant steroid. Immunohistochemical studies have shown that both 3βHSD (113) and $P450_{arom}$ (93) are present in TICs from selectable follicles, but that both $P450_{scc}$ and $P450_{17\alpha}$ are absent (111, 112). The presence of A in follicular fluid (FF), however, suggests that these latter enzymes are probably insufficiently expressed to be detected by immunohistochemical methods. Although the percent conversion rate of A to E_2 by cultured GCs increases slowly when the follicle diameter exceeds approximately 3 mm (110), $P450_{arom}$ has not been detected in most of the studies (111, 112, 118, 119). These observations, therefore, indicate that selectable follicles become more responsive to gonadotropins in terms of quality and growth rate but that their FSH-induced aromatase remains poorly expressed.

3. *The newly selected follicle.* During the early follicular phase, just after having been selected, the follicle destined for ovulation contains detectable levels of FSH (120), and the mitotic index of its GCs is very high (60). Whereas A was largely predominant in FF of selectable follicles, its concentration decreases in newly selected follicles while the E_2 concentration strongly increases as compared with selectable follicles, leading to a low FF A/E_2 ratio (Table 3). This is due to $P450_{arom}$ that is expressed in GCs from follicles of a similar size (111). These data, therefore, indicate that during selection, the follicle destined for ovulation changes from an androgen- to an estrogen-producing structure by expressing its FSH-induced aromatase activity.

4. *Maturation of the preovulatory follicle.* In parallel with its increasing size, high proliferative activity of GCs, and morphological alterations of the theca and granulosa layers, the preovulatory follicle undergoes marked changes in steroidogenic activity as its maturation progresses. Enzymes such as 3βHSD, $P450_{17\alpha}$, and $P450_{scc}$ are detected at a significant level in TICs from these follicles (111–113, 121), and $P450_{arom}$ enzyme exhibits strong staining in GCs when the follicular diameter is more than 11 mm (93, 112). Significant levels of mRNAs, for $P450_{SCC}$ and $P450_{17\alpha}$ appear in TICs of preovulatory follicles from D7 and D8, respectively (119). The levels of mRNAs for $P450_{arom}$, which were low on D8 and D9, strongly increase on D13 (119). Consequently, steroid production increases strongly, and from the early to late follicular phase before the midcycle gonadotropin surge, E_2 in FF increases from around 658 to approximately 2,396 ng/ml, and mean concentration of steroids increases from 2,275 to 4,829 ng/ml (Table 3). These studies are in keeping with early biochemical studies indicating that aromatase activity increases progressively during the preovulatory phase, reaching 200 times that found in cells obtained from selectable follicles (94).

Thus, during the follicular phase, GCs from the selected follicle exhibit an increasing aromatase activity. Since circulating levels of FSH are high at this time, it can be assumed that GCs of the early preovulatory follicle become fully responsive to FSH.

The full responsiveness of preovulatory GCs to FSH is also confirmed by progressive acquisition during preovulatory maturation of other FSH-induced functions such as the expression of LH-R, which is induced under the primary control of FSH in most species (122). During preovulatory maturation a dramatic increase in LH binding occurs in human GCs (123, 124). Once they have synthesized LH-R, preovulatory GCs become able to respond to the midcycle gonadotropin surge. During the late follicular phase, after the gonadotropin surge, the mean concentration of steroids in FF increases from 4,829 to 10,995 ng/ml (Table 3). From the time of the gonadotropin surge, GC proliferation becomes extremely low and FF concentrations of E_2 and A decrease, whereas those of P and 17α-hydroxyprogesterone (17α-OHP) increase sharply (Table 3). Concomitantly, 3βHSD detected both by enzymohistochemistry (121) and immunolocalization (113) exhibits strong activity in GCs.

Thus, when folliculogenesis is completed, just before ovulation, GCs are highly differentiated in the preovulatory follicle, having stopped to proliferate but producing high levels of steroids.

In conclusion, the analysis of folliculogenesis indicates that follicular responsiveness to gonadotropins increases progressively as the follicle develops from the preantral to preovulatory stage. How this occurs in primates remains poorly

TABLE 3. Changes in FF mean (±SEM) concentrations (ng/ml) of steroids from the selectable to the preovulatory stage

Follicle type	E_2	A + T + DHT	17α-OHP	P	Total
Atretic (1–5 mm)	20 ± 5	794 ± 90		73 ± 13	887
Selectable	15 ± 5	638 ± 113		130 ± 45	783
Newly selected (EF)	658 ± 38	487 ± 128	713 ± 318	417 ± 120	2,275
Preovulatory (MF)	1,270 ± 161	542 ± 176	460 ± 112	440 ± 74	2,712
Preovulatory (LF:E_2 peak)	2,396 ± 348	203 ± 37	1,002 ± 212	1,228 ± 228	4,829
Preovulatory (LF:E_2<<LH surge)	2,583 ± 228	287 ± 44	1,812 ± 142	2,464 ± 226	6,146
Preovulatory (after the LH surge)	1,109 ± 142	79 ± 21	2,034 ± 326	7,773 ± 643	10,995

Mean concentrations are estimated from Refs 61, 115–117.
EF, Early follicular phase; MF, midfollicular phase; LF, late follicular phase.

understood but suggests specialized modifications at the cellular level. The wealth of information existing on ovarian responsiveness obtained through *in vitro* and *in vivo* studies may provide clues as to the nature of the intraovarian events responsible for the increase in sensitivity to gonadotropins. We will review the existing data in the following section.

V. Factors Regulating the Progressive Acquisition of Follicular Responsiveness to Gonadotropins

A major question in this issue may be: is the acquisition of follicular responsiveness to gonadotropins solely attributable to changes in gonadotropin receptor content or function, or is it also attributable to molecules acting at the level of follicular cells?

A. The gonadotropin receptors

Gonadotropins act on follicular cells by coupling with receptors localized at the cell surface. FSH binds exclusively to FSH-R in GCs, whereas LH binds to LH-R, initially localized to TICs, and then to GCs during maturation of the preovulatory follicle (125).

FSH-Rs have been detected on GCs of human follicles from the secondary stage onward (123, 124). Despite the absence of data on the precise stages at which FSH-Rs increase in number, the density of FSH binding to GCs is higher in antral than preantral follicles and does not vary when antral follicles grow from 3–12 mm (124). However, human preovulatory GCs in the late follicular phase bind less FSH than preovulatory GCs in the midfollicular phase (124). Despite the synthesis of FSH-R on GCs being modulated by several factors, the process has long been recognized to be under the primary control of FSH (126). Yet, a recent study indicated that in *hpg* mice lacking circulating gonadotropins, the ovaries contain FSH-R mRNAs, which suggests that factors other than FSH act on the expression of the FSH-R gene (42).

Nonovulatory follicles in both monkeys (127) and humans (128) do not possess LH-R on their GCs since hCG does not stimulate their steroidogenesis. Receptors for LH appear in preovulatory GCs starting in the midfollicular phase (124, 127) and are in highest density in mural GCs located next to the basal lamina (124).

TICs possess LH-R at all stages from the preantral stage onward as demonstrated by autoradiographic and immunocytochemical studies in monkeys (69) and humans (123, 124). The LH binding to primate TICs starts with the appearance of epithelioid cells and increases during follicular maturation. Binding is more important in TICs of 3-mm follicles than in preantral or early antral follicles and is stronger in 4- to 7-mm as compared with 2- to 3-mm follicles (124).

Taken together, these data indicate that the number of LH-Rs increases in TI, and that of FSH-Rs increases first, then decreases as the primate follicle develops. Nevertheless, data concerning the number of FSH-Rs on GCs remain imprecise, especially at times when an increasing follicular responsiveness to gonadotropins has been detected. Further studies are therefore required to analyze the dynamic changes of GC content in FSH-Rs and their mRNAs at each critical stage of follicular development. However, one question must be addressed: do changes in the number of gonadotropin receptors or in the levels of their mRNAs account for the changes of follicular responsiveness to gonadotropins during follicular growth?

In some cases, the detection of mRNAs for gonadotropin receptors cannot explain these changes. Because there are multiple transcripts and splice variants encoding the LH-R and FSH-R, it is possible that some of them do not exhibit functional activity (Ref. 122 and references therein). Hence, it has been hypothesized that increasing levels of FSH-R mRNAs without an increase in active membrane receptors (129) may be caused by the transcription of an inactive splice variant and not the expression of mRNAs encoding the functional receptors (122, 129).

Added to the complexity of the expression of the LH-R and FSH-R genes and multiple forms of mRNAs are the transducing mechanisms to which these receptors couple. In rats, GCs isolated from preovulatory follicles exhibit greater FSH-responsive adenylyl cyclase than GCs isolated from small antral follicles in which steroidogenesis is low (130), despite the similar numbers of FSH-binding sites in the two GC populations (131). In porcine GCs, FSH enhances steroidogenesis at concentrations that cause a decrease in the number of FSH-Rs, thereby suggesting either that only a small number of FSH-Rs are required to elicit a response in target cells or that less FSH is needed once the biosynthetic pathway is activated (129). These observations have raised the possibility that there are different transducers to which FSH-R can couple or that adenylyl cyclase activity can be modified. Recent studies suggest that FSH-R and LH-R present at the early stages of follicular development may couple to subsets of transducers different from those to which they couple at later stages, which could lead to different physiological effects (122).

Mechanisms regulating the gonadotropin receptor gene expression and the transducing mechanisms to which these receptors couple are highly complex and unknown in primate follicle cells. Consequently, the precise role that gonadotropin receptor may play to increase the gonadotropin responsiveness of follicular cells during human folliculogenesis remains unelucidated and is a promising field of investigation.

Irrespective of possible changes in levels or function of FSH-R, many experiments performed in mammals suggest that FSH action on GCs can be modulated by biological substances present in serum or synthesized by the follicle itself. Although FSH is required to promote normal follicular development, no growth-promoting effect of FSH has been found on GCs from either small or large follicles cultured *in vitro* without serum in rats (132) and cattle (133). But, in the presence of serum (133, 134), FSH is an effective stimulator of cell proliferation. Conversely, GC differentiation only occurs in absence of serum (135, 136). These data, therefore, suggest the existence of factors in serum inhibiting GC differentiation and promoting GC proliferation.

On the other hand, FSH acts on preantral human follicles cultured *in vitro* without serum to stimulate the proliferation of GCs, to induce the differentiation of an antral cavity, and to stimulate the production of E_2, P, and A (108). Similarly, FSH stimulates DNA synthesis of GCs in incubated whole follicles in hamsters (137) and mice (138) without serum or supplemented milieu, suggesting that follicular tissues, GCs

and/or TICs, produce substances acting in an autocrine or paracrine fashion, alone or in synergy with FSH to stimulate GC proliferation and differentiation.

Altogether, these observations lead to the hypothesis that *in vivo* FSH-induced functions in growing follicles may be mediated by factors coming from the serum or from the follicle itself, acting alone or in synergy with FSH.

B. The intraovarian peptides

There is evidence that the effect of gonadotropins on follicular cell proliferation and cytodifferentiation is modulated by nonsteroidal products secreted by GCs and/or TICs. Growth factors have been recognized as peptides that may be involved in cell-cell interactions in the follicle.

1. The insulin-like growth factor (IGF) system. The IGF system consists of IGF-I and IGF-II, type I (IGF-I-R) and type II (IGF-II-R) receptors, and a family of binding proteins (IGFBPs) that modulate the actions of IGFs (for review see Ref. 3).

a. The IGF system in humans. In humans, IGFs alone possess stimulatory effects on both proliferation and steroidogenesis in GCs and TICs from immature to preovulatory follicles (Table 4), and these effects are strongly enhanced by the presence of gonadotropins.

The presence of IGFs and their receptors, as well as their respective mRNAs, has been reported in human follicles at successive stages of their development (Table 5). Data are sometimes confusing. Whereas IGF-I and its mRNA have not been detected in GCs, their presence in TICs is disputed. IGF-I has been transiently detected in TICs from antral nonovulatory follicles by Hernandez *et al.* (160), but its mRNA has not been found by Zhou and Bondy (162). Conversely, El Roeiy *et al.* (161) found IGF-I mRNA but not IGF-I in TICs from follicles of similar size. There are several explanations to account for this disparity between gene expression and the absence of the gene product, including rapid secretion or the inability of the antiserum used to detect the small amount of protein present. Similarly, the production of IGF-II and its mRNA by nonovulatory follicles is unclear. El Roeiy *et al.* (161) have detected IGF-II and its mRNA only in preovulatory follicles, thus confirming the previous observation that cultured GCs sampled in women undergoing IVF & ET (IVF-GCs) can synthesize IGF-II (164). However, Zhou and Bondy (162), who have not studied preovulatory follicles, found IGF-II mRNA in GCs from small growing follicles. Data reported in Table 5 suggest that the human follicle is a site of IGF-II rather than of IGF-I production. The situation prevailing in the human ovary appears to be different from that of the rat ovary, in which IGF-I gene expression is restricted to GCs (165).

TABLE 4. Reported effects of IGFs on human follicle cells

Granulosa
IGF-I, alone or in synergy with FSH, stimulates proliferation of immature GC, mature GC, and IVF-GC (139–142)
IGF-I, alone or in synergy with FSH, stimulates aromatase mRNA levels and aromatase activity in immature GC, mature GC, and IVF-GC (141, 143–146)
IGF-I alone stimulates P production, independently of cell maturity, when GC are cultured in presence of calf serum (141, 144). In serum free conditions IGF-I alone is without effect (147)
IGF-I enhances FSH- and/or LH-induced P production independently of cell maturity (141, 144, 147)
IGF-II alone stimulates both E_2 and P production by IVF-GC when they are cultured in presence of calf serum (148)
Theca interna
IGF-I stimulates TIC proliferation in culture (149)
IGF-I strongly enhances cAMP-stimulated production of 17α-OH P and $P450_{17\alpha}$ (150)
IGF-I and IGF-II, alone or in synergy with LH and inhibin, strongly stimulate A production by TIC (89, 151)
In synergy with LH, IGF-I enhance nearly 4-fold A secretion and on average 2.4 times T secretion by TIC (151)
IGF-II, alone or in synergy with LH, stimulates A production (89)

Under normal physiological conditions, the IGFs in body fluid are bound to a family of homologous proteins termed the IGF-binding proteins (IGFBPs) (for review see Ref. 3). Six different IGFBPs have been detected in the human ovary. IGFBP-I, -2, -3, and -4 have been detected in the fluid of follicles from 3 mm in diameter to the preovulatory stage (146, 153, 157, 166–172), and IGFBP-6 has been found in FF of stimulated preovulatory follicles (173). According to the stage of follicular development, GCs contain mRNAs encoding IGFBP-1, -2, -3, -4, and -5 (162, 168, 174) and cultured IVF-GCs synthesize IGFBP-1, -2, -3, and -4 (168, 175–178). At all stages of follicular development, IGFBP-2 mRNA levels are high in TICs (161), which also secrete IGFBP-3 in culture (175).

There are consistent changes in levels of certain IGFBPs in association with the stage of follicle development. IGFBP-1 appears at higher levels in follicles after LH/hCG (167) and have been associated with luteinization of GCs (166–168), and levels of both IGFBP-2 and -4 strongly decrease in preovulatory follicles (170–172).

Although a local amplifying effect of IGFBPs on IGF action has been suggested (179), it is more generally admitted that IGFBPs remove free IGF from follicular environment and, by preventing its binding to receptors, restrict IGF action on ovarian function (153). In humans, IGFBP-1 inhibits IGF-I-mediated proliferation of IVF-GCs (140), and both IGFBP-1 and IGFBP-3 inhibit IGF-I-stimulated E_2 and P productions by these GCs (180). Moreover, in rats, all substantial inhibitions of FSH action induced by IGFBPs can be mimicked by anti-IGF-I antibody (181).

b. The GH/IGF axis. GH has recently been viewed as a "co-gonadotropin" capable of enhancing gonadotropin action at the ovarian level (182). GH and GH-releasing hormone (GHRH) have been reported to improve the effects of treatments with gonadotropins in patients undergoing IVF & ET whether normal or poor responders (183–185), in women with anovulation and amenorrhea (186) or suffering from a PCOS (183), and in hypogonadotropic subjects (187). However, these observations are disputed. Other studies have shown no beneficial effect of GH administered to poor or normal responder IVF patients treated with similar doses of hMG (188–190), and GHRH injected from cycle day 2 to the time of ovulation does not increase the number of fertilized oocytes and resulting pregnancies (191). In addition, in an infertile patient suffering from a GH deficiency (Oliver-McFarlane syndrome), adjuvant GH did not influence the ovarian response to exogenous gonadotropins (192).

TABLE 5. Follicular levels of IGF-I, IGF-II, IGF-I-R, IGF-II-R, and their respective mRNA (in parentheses) in human follicles

Stage of follicle growth	Granulosa			
	IGF-I	IGF-II	IGF-I-R	IGF-II-R
Primary	(−)		++	
Secondary	(−)	(−/+)	++	
Preantral (class 1)	(−)	(−/+)	++ (+/++)	
Small antral (classes 2–4)	− (−)	− (−/+)	+/++ (+/++)	+ (++)
Selectable (class 5)	− (−)	− (−/+)	+/++ (+/++)	+ (++)
Preovulatory (classes 6–8[a])	− (−)	+ (+++)	+/++ (++)	+ (+++)
Preovulatory (class 8[b])	− (−)	+ (+++)	+/++ (++)	+ (+++)
Stage of follicle growth	**Theca interna**			
	IGF-I	IGF-II	IGF-I-R	IGF-II-R
Primary				
Secondary				
Preantral (class 1)	(−)		+	
Small antral (classes 2–4)	− (−/++)	+ (++)	++ (+/++)	+ (+)
Selectable (class 5)	− (−/++)	+ (++)	++ (+/++)	+ (+)
Preovulatory (classes 6–8[a])	− (−)	− (−)	++ (+/++)	+ (+)
Preovulatory (class 8[b])	− (−)	− (−)	++ (+/++)	+ (+)
Stage of follicle growth	**Follicular fluid**			
	IGF-I	IGF-II		
Selectable (classes 5 and 6)	+			
Preovulatory (classes 6–8[a])	++	+++		
Preovulatory (class 8[b])	++	+++		
Stage of follicle growth	**Oocyte**			
	IGF-I	IGF-II	IGF-I-R	IGF-II-R
Primary	(−)	(++)	+++ (+++)	
Secondary	(−)	(++)	+++ (+++)	
Preantral (class 1)	(−)	(++)	+++ (+++)	
Small antral (classes 2–4)	(−)	(++)	+++ (+++)	
Selectable (classes 5 and 6)	(−)	(++)	+++ (+++)	
Preovulatory (classes 6–8[a])				
Preovulatory (class 8[b])				

The staining or the level in FF was determined as positive (+ to +++) or negative (−).
[a] Selected follicle before the midcycle gonadotropin surge.
[b] Preovulatory follicle after the spontaneous midcycle gonadotropin surge or after an hyperstimulation treatment.
[Data from Refs. 48, 148, and 151–163].

If it exists, the nature of GH action at the ovarian level remains unclear despite GH being the primary regulator of serum IGF-I levels (3). In women treated with hMG and GH, IGF-I concentrations rise in FF (183) and serum (186) and are significantly higher in GH-treated women as compared with controls. However, IGF-I FF levels are significantly lower than serum levels (183, 186), and since GH is incapable of altering IGF-I and IGF-II mRNAs in cultured IVF-GCs (158), the source of increasing circulating IGF-I is presumed to be the liver. Thus, GH action might be indirect by stimulating hepatic IGF-I, which can act alone or improve gonadotropin action at the follicular level. Alternatively, GH may exert a direct effect on GCs since GH receptors and their mRNAs have been identified in GCs from nondominant and/or dominant follicles in natural or stimulated cycles (182, 193–195).

Taken together, the available data indicate that uncertainties remain regarding the role of GH in enhancing follicular growth. Spontaneous and induced pregnancies have been reported in two women with Laron-type dwarfism, a condition characterized by a deficiency of GH receptors and subsequent systemic IGF deficiency (196, 197). This suggests that while circulating IGF-I and/or GH may contribute, their role is not indispensable for normal folliculogenesis in humans.

In conclusion, available data indicate that IGFs may play a role in regulating both proliferation and steroidogenesis of human follicles by amplifying the stimulatory signals of FSH and LH in growing and preovulatory follicles. This impact of IGFs on both GCs and TICs argue in favor of the ovarian IGF-II system playing a key role in human ovarian follicular growth, whereas the role of IGF-I in regulating human follicular function is as an endocrine hepatic hormone rather than a paracrine/autocrine factor of follicular origin.

2. Epidermal growth factor (EGF) and transforming growth factor-α (TGFα). EGF and TGFα are polypeptides that have structural homology and bind to a common EGF/TGFα receptor (EGF-R) (for review see Ref. 198).

In humans, EGF stimulates *in vitro* proliferation of both immature and IVF-GCs and inhibits FSH-stimulated E_2 synthesis in cultured GCs (Table 6). The ability of EGF to enhance both basal and hCG-stimulated P production in cultured IVF-GCs (200, 209) has not been confirmed by other

TABLE 6. Effects of EGF/TGFα at the follicular level

Granulosa
Stimulates proliferation (human follicles) (199, 200)
Attenuates FSH-induced FSH-R mRNA expression (rat) (201)
Attenuates activin-induced FSH-R synthesis (rat) (202)
Attenuates FSH-induced LH-R synthesis (rat, pig) (203, 204)
Inhibits FSH-induced estradiol synthesis in immature GC (205)
Inhibits FSH-induced aromatase expression in IVF-GC (206)
Inhibits inhibin secretion (bovine) (207)
Theca interna
Inhibits cAMP-induced production of 17α-OH P and DHEA, and cAMP-stimulated P-$450_{17\alpha}$ activity (150)
Inhibits LH-induced androgen synthesis (rat) (208)
Dedifferentiates theca cells into stroma cells (rat) (208)

When available, the reported effects come from experiments performed in primates; when not available in primates, the reported effects come from other mammal species and are in *italics*

studies (150, 210). The reason for this is probably that the authors used different culture conditions.

Both EGF (52) and TGFα (51), as well as EGF-R, have been immunohistochemically detected in human follicular tissues (Table 7). Their immunostaining increases as follicular size increases. However, these findings are not in keeping with the observed decrease in EGF and TGFα levels seen in FF as follicles enlarge (211–214). Hence, intrafollicular concentrations of EGF decrease approximately 3-fold when follicles grow from 1 to 6 mm (212), and those of TGFα decrease more than 10-fold when follicles grow from 2 to 10 mm (214). In 6-mm follicles, EGF concentration is 2-fold higher than in serum (211, 212) whereas it is half the serum value in preovulatory FF (211).

Taken together, available data support the view that in humans, EGF and TGFα are negative modulators of FSH-induced synthesis of E_2 and positive modulators of GC proliferation. Their production by human follicles seems to decrease as follicular diameter enlarges.

3. Basic fibroblast growth factor (bFGF). bFGF is a peptide that has been isolated from a wide range of tissues. It is known to be mitogenic for GCs in a variety of species, including humans (199), to play an inhibitory action on GC differentiation (150, 215, 216) and TIC steroidogenesis (150), and to be a potent angiogenic factor (217). In humans, mRNAs encoding for bFGF and its receptor have been observed in the fetal ovary (218) and in IVF-GCs (219, 220). The RT-PCR technique used to detect these mRNAs in the latter cells indicates that the message is very low. To date, the possible role of bFGF in human follicular function has received little attention.

4. TGFβ. TGFβ is a homodimeric polypeptide with at least three isoforms (TGFβ1, TGFβ2, and TGFβ3) that have been identified in mammalian cells (for review see Ref. 198).

There are few available data concerning the action of TGFβ on follicular tissues in primates. TGFβ inhibits forskolin-induced P$450_{17\alpha}$ activity, and 17α-OHP and dehydroepiandrosterone production by cultured TICs from human preovulatory follicles, but enhances forskolin-stimulated 3βHSD activity by these cells (150). At the level of GCs, TGFβ alters neither aromatase activity nor P production (150). In addition, TGFβ enhances inhibin production by rat GCs (221), as well as binding of FSH to GCs (222).

In humans, TGFβ1 and TGFβ2 have been immunohistochemically detected in both GCs and TICs of growing follicles from the early antral stage (Table 8). The lack of detectable TGFβ in the FF, as shown in the porcine (225), suggests that TGFβ actions may be only local.

Hence, available data in humans support the view that TGFβ production is predominantly associated with the largest follicles and that TGFβ may favor production of P by TICs.

5. Inhibin, activin, and follistatin (FSP). Inhibin is a glycoprotein that has been isolated from FF as a heterodimer consisting of a common α-subunit combined with one of two β-subunits, βA (inhibin-A) or βB (inhibin-B). The βA- and βB- subunits give rise to the three forms of activins: activin (βAβB), activin A (βAβA), and activin B (βBβB). FSP is a monomeric glycosylated protein that is the major high-affinity-binding protein for both inhibin and activin present in human FF (for review see Ref. 226).

Among the most remarkable effects of inhibin and activin, are the opposite effects that these peptides exert on certain follicular functions (Table 9). On one hand, inhibin stimulates and activin inhibits androgen production by TICs. On the other hand, inhibin may be a potent inhibitor of ovarian cell proliferation in mice (236), while activin has been shown to stimulate *in vitro* proliferation of immature rat GCs (237) and of human IVF-GCs (229). However, the possible role of activin in regulating aromatase activity in primate GCs remains unclear because activin stimulates FSH-responsive aromatase activity in GCs from marmoset

TABLE 7. Intrafollicular levels of EGF, TGFα, and EGF-R during folliculogenesis in primates

Follicle	EGF				TGFα				EGF-R		
	GC	TIC	FF	O	GC	TIC	FF	O	GC	TIC	O
Primary				+	+			+			+
Secondary				+	+			+			+
Preantral (class 1)				+	+	+		+			+
Small antral (classes 2–4)	+	+	+++	++	++	+			+	+	+
Selectable (classes 5 and 6)	++	++	+++	++	++	+/++	+++		++	++	++
Preovulatory (classes 6–8[a])	++	++	+	++	+++	++	+		++	++	++
Preovulatory (class 8[b])	++	++	+		+++	+++			++	++	

The staining or the level in FF was determined as positive (+ to +++).
[a] Selected follicle before the midcycle gonadotropin surge.
[b] Preovulatory follicle after the spontaneous midcycle gonadotropin surge or after an hyperstimulation treatment.
FF, Follicular fluid; O, oocyte. [Data from Refs. 50–52, and 211–214.]

TABLE 8. Follicular levels of TGFβ during the primate folliculogenesis

Follicle type	TGFβ1		TGFβ2		TGF-β1 and β2 mRNA
	GC	TIC	GC	TIC	GC
Small antral (classes 2–4)	−/+	+		+	
Selectable (classes 5 and 6)	+	++		++	
Preovulatory (classes 6–8[a])	++	+++	+	+++	
Preovulatory (class 8[b])	++	+++	+	+++	+

The staining was determined as positive (+ to +++) or negative (−).

[a] Selected follicle before the midcycle gonadotropin surge.

[b] Preovulatory follicle after the spontaneous midcycle gonadotropin surge or after an hyperstimulation treatment.

[Data from Refs. 223 and 224.]

monkeys and inhibits aromatase activity in human IVF-GCs (231). Activin has been shown to play an important role in regulating the functional integrity and development of GCs in the ovarian follicle. Activin increases the number of FSH-Rs in rat GCs (202, 232) and induces reorganization of follicular structures from monolayer culture of immature GCs in both rats and humans (237). Receptors for activin as well as their mRNAs have been localized in oocytes, GCs, and TICs of the rat (238, 239). However, although activin exhibits many effects on human follicular cells, mRNAs for type II activin receptors (Act RII and Act RIIB) have not been detected in any cell layer in 3- to 7-mm human follicles (240).

The FSP effects on P production by preovulatory GCs are indirect. FSP inhibits the suppressive effects of activin (230), since it binds with high affinity to activin and with lower affinity to inhibin (241, 242).

In primates, activin (234), inhibin, inhibin subunits, and FSP (Table 10), as well as their respective mRNAs (240, 245, 246, 250, 251), have been predominantly detected in GCs. However, recent findings indicate that inhibin subunits, FSP, and their respective mRNAs may not be exclusively synthesized by GCs. While α-subunit mRNA was detected in TICs from 1- to 8-mm human follicles, there was no immunoreactivity for the protein. Likewise, βA-subunit and FSP immunoreactivity were localized in TICs from 1- to 8-mm follicles with no corresponding detection of mRNA signals (240, 250), whereas βA-subunit mRNA was detected in TICs from dominant follicles, but the protein was not found in these cells (250). These discrepancies may be explained by several mechanisms (240) but need to be confirmed by further studies.

The kinetics of production of inhibin subunits in primate GCs during folliculogenesis indicate that the activin gradient of the developing follicle declines as its inhibin and FSP gradient increases. Although the *in vivo* roles of these peptides are still incompletely understood, these changes could have implications for their putative regulatory functions during folliculogenesis.

6. Cytokines. Cytokines, which are primarily produced by white blood cells, constitute a group of proteins that modulate a variety of cellular functions (for review see Refs. 252–254).

In humans, various ovarian effects of cytokines on cultured IVF-GC functions have been reported (Table 11). Taken together, available data indicate that cytokines may be inhibitors of GC steroidogenesis just before ovulation. Some cytokines could alter gonadotropin-stimulated production of steroids via specific receptors because receptors for interleukin-1 (IL-1) and their mRNAs have been detected in IVF-GCs (263, 264).

Tumor necrosis factor-α (TNFα) (255, 265), interferon-α and -γ (266), IL-1, IL-2 (260, 267, 268), and IL-6 (269, 270) have been identified in FF from women undergoing IVF&ET. Levels of interferon-α and -γ in FF do not correlate with serum values whereas those of both IL-1 and IL-2 are half the serum value, and those of IL-6 are 3- to 30-fold higher than levels detected in serum.

Messenger RNAs for IL-1β are absent from whole ovarian material from days 4 or 12 of an unstimulated menstrual cycle, but IL-1α and IL-1β mRNAs have been detected in preovulatory follicular aspirates obtained at the end of an IVF cycle (263, 264). Whether IL-1β and IL-1α mRNAs are produced by white blood cells, or by GCs, or both, remains unclear. White blood cells are present in large numbers in the human follicle wall at the time of ovulation, and IVF-GCs contain 5–15% of macrophages and monocytes (271, 272). This is because soon before ovulation, after the basal lamina is disrupted, thecal capillaries rapidly infiltrate the ovarian granulosa layer and come into direct contact with GCs. In addition, a protein factor that is chemotactic for neutrophils was found in human FF (273) and may facilitate infiltration of granulosa layer by these cells. Yet, when the population of monocytes is reduced by using various processes (260, 263), the "pure" GC population still contains IL-1β. Since peripheral monocytes obtained at the time of oocyte retrieval are negative for IL-1β gene expression (263) and because IVF-GCs, not macrophages, account for the majority of the immunocytochemical staining for IL-1β in follicular aspirates (264), it has been suggested that IL-1β may be derived from GCs. Similarly, a recent study led to similar conclusions concerning IL-6 (269). In the human ovary, TNFα has been detected by immunohistochemistry in healthy antral follicles at the level of the antral layer of GCs (265). Because FSH has been shown to increase release of TNFα by cultured IVF-GCs possibly contaminated by macrophages, the effects of FSH on release of TNFα has been tested directly on macrophages (258). No effect of FSH was observed on these latter cells, but it is possible that FSH indirectly induces the release of TNFα. Thus, some evidence supports the view that GCs may synthesize IL-1, IL-6, and TNFα.

In conclusion, although cytokines generally inhibit gonadotropin-induced steroidogenesis in cultured IVF-GCs, the physiological implications of their action remain unclear. Espey (274) has noted that ovulation involves mechanisms common to an inflammatory reaction, leading to the hypothesis that cytokines may also have a role in ovulation and luteinization rather than in cellular growth and differentiation during folliculogenesis. This hypothesis is strengthened by the observation that most cytokines appear in human follicles only a short time before ovu-

TABLE 9. Reported effects of inhibin, activin, and FSP at the follicular level

	Inhibin
Granulosa	Slightly inhibits FSH-induced aromatase (marmoset antral follicle) (227)
Theca interna	Strongly enhances IGF-I- and LH-induced androgen production (human antral follicle) (228)
	Inhibits activin-induced suppression of theca cell androgen synthesis (human antral follicle) (149, 227)
	Activin
Granulosa	Stimulates proliferation in cultured IVF-GC (229)
	Enhances FSH-induced aromatase expression (marmoset antral follicle) and basal and LH-induced aromatase expression (marmoset preovulatory follicle) (227)
	Inhibits LH-induced progesterone production (marmoset and human preovulatory follicles) (227, 230)
	Decreases, in a dose-related fashion, P production and aromatase activity in cultured IVF-GC (231)
	Increases the number of FSH-R (rat) (202, 232)
	Stimulates FSH-stimulated LH-R synthesis (rat) (233, 234)
Theca interna	Strongly inhibits IGF-I- and LH-induced androgen production (149)
	FSP
Granulosa	Increases basal progestin production (human preovulatory follicle) (235)
	Mainly inhibits activin actions (human preovulatory follicle) (235)

When available, the reported effects come from experiments performed in primates; when not available in primates, the reported effects come from other mammal species and are in *italics*.

TABLE 10. Follicular levels of inhibin, inhibin subunits, and FSP during primate folliculogenesis

Follicle type	Inhibin		α-subunit	βA-subunit	βB-subunit	FSP
	GC	FF	GC	GC	GC	FF
Small antral (classes 2–4)	−/+	+	−	++	+++	
Selectable (classes 5 and 6)	+/++	+	−/+	++	+++	++
Preovulatory (classes 6–8[a])	++/+++	++	++	++	−/+	++
Preovulatory (class 8[b])	+++	+++	++	++	−/+	+++

The staining or the level in FF was determined as positive (+ to +++) or negative (−).
[a] Selected follicle before the midcycle gonadotropin surge.
[b] Preovulatory follicle after the spontaneous midcycle gonadotropin surge or after an hyperstimulation treatment.
FF, Follicular fluid. [Data from Refs. 243–250.]

TABLE 11. Effects of cytokines on human IVF-GC

TNF-α
Simulates proliferation (255, 256)
In synergy with IFNγ or IL-1, inhibits hCG-induced P production (257)
In synergy with IFNγ, reduces FSH-induced E_2 production (257)
Simulates $PGF2_\alpha$ production (255, 258)
Interleukins
IL-1 inhibits FSH- and hCG-stimulated E_2 production (259, 260)
IL-2 inhibits hCG-stimulated P production (261)
Interferons
Reduce the levels of E_2 and P in FF (IVF follicle) (262)
IFN-α, -β, and -γ reduce hCG-stimulated P secretion and FSH-stimulated E_2 (257, 261)
When administered *in vivo*, IFNα reduces FF and serum levels of E_2, and IFNγ reduces FF and serum levels of P, in the late follicular phase without affecting gonadotropin levels (262)

lation and by studies showing that cytokines display stimulating effects on various ovulation-associated phenomena (255, 258, 264, 274).

VI. Atresia

In mammals, it has long been recognized that the majority (>99%) of follicles present at birth become atretic with only less than 1% achieving ovulation. Thus, the "normal" fate for each individual follicle is to disappear by entering atresia, a process that may be considered normal, allowing the ovary to produce cyclically the ovulatory quota.

A. Morphological characterization of atresia

In histological studies, morphological criteria have been used to assess the health status of individual follicles (*i.e.* whether actively growing or degenerating). Oocyte involution, irregular shape of the follicle and/or of the oocyte and, above all, nuclear pyknosis in granulosa layers (Fig. 8) are criteria most commonly used to describe early atresia (275). It is now accepted that GC pyknosis is an apoptotic process (276–278). As a result of endonuclease activity, genomic DNA is cleaved into nucleosomal oligomers of about 180-bp nucleosomal units before apoptosis becomes morphologically evident (for review see Ref. 278). The first morphological evidence of apoptosis is condensation of nuclear chromatin into crescentic caps at the periphery of the nucleus, an effect associated with nucleolar disintegration and an overall reduction in nuclear size. At the same time, cytoplasmic condensation results in a reduction of total cell volume and a related increase in cell density.

Atretic human follicles larger than 1 mm have been arranged into four successive stages of degeneration (Fig. 8). Whereas in follicles less than 1 mm, atresia is characterized by a fast and early oocyte degeneration as well as hypertrophy of TICs (Fig. 9), the oocyte persists long after GCs have

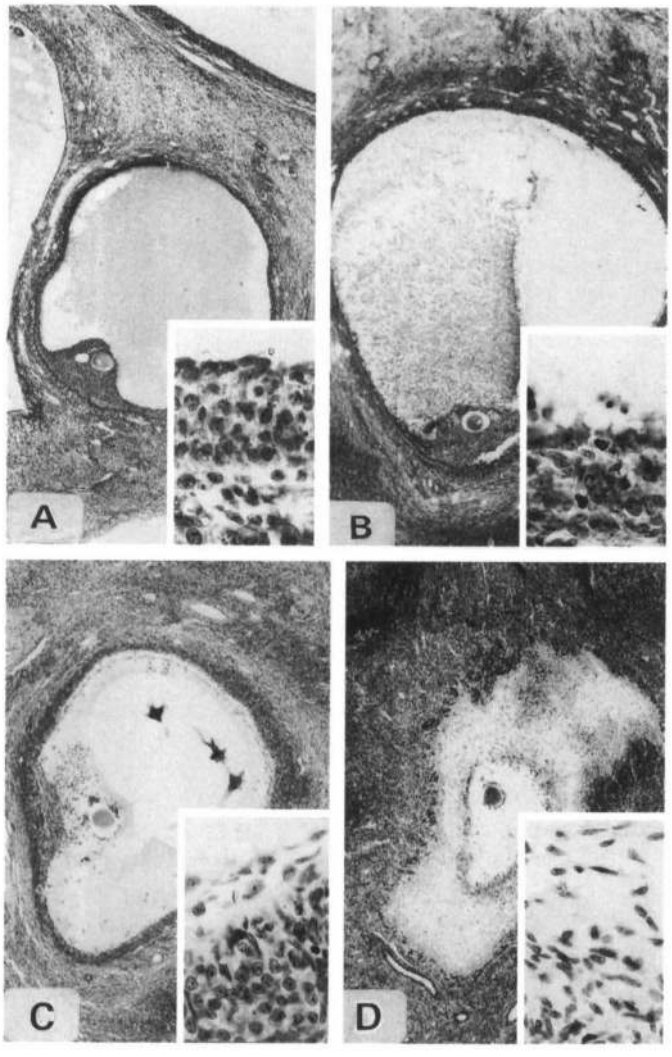

FIG. 8. Atresia in human antral follicles larger than 1 mm. A, Stage A: up to 20% of the GC of the earliest atretic follicles exhibit pyknoses and fragmentation. Theca and granulosa layers are not markedly altered, and the follicular shape remains regular with a normal basal lamina, ×30, ×320. B, Stage B is a further step of atresia, pyknotic GCs are numerous, and 10–50% of the granulosa wall has disappeared. The TI is not altered, and the follicular shape remains regular, ×30, ×320. C, Stage C: the follicle is shrunken, and the cumulus oophorus persists despite the disappearance of the GCs. TI has changed into an interstitial gland, and basal lamina is thickened with a thin strip of fibroblasts in its inner part, ×30, ×320. D, Stage D corresponds to the ultimate stage of degeneration; the follicle is more or less collapsed, and granulosa, theca-interstitial gland, and cumulus oophorus have disappeared. The basal lamina is very thick and the antrum is invaded by fibroblasts. When not degenerated, the oocyte is floating free in the antral cavity, ×30, ×320. [Reproduced with permission from A. Gougeon, *The Ovary*, pp. 21–39 (5).]

disappeared in follicles larger than 1 mm and displays cyclic meiosis-like alteration (including germinal vesicle breakdown, alignment of chromosomes in metaphase, and expulsion of a polar body) that rises sharply during the periovulatory period (279).

B. Follicular atresia in the human ovary during the menstrual cycle

It is generally assumed that adult follicular atresia is composed of an underlying tonic component that mainly affects

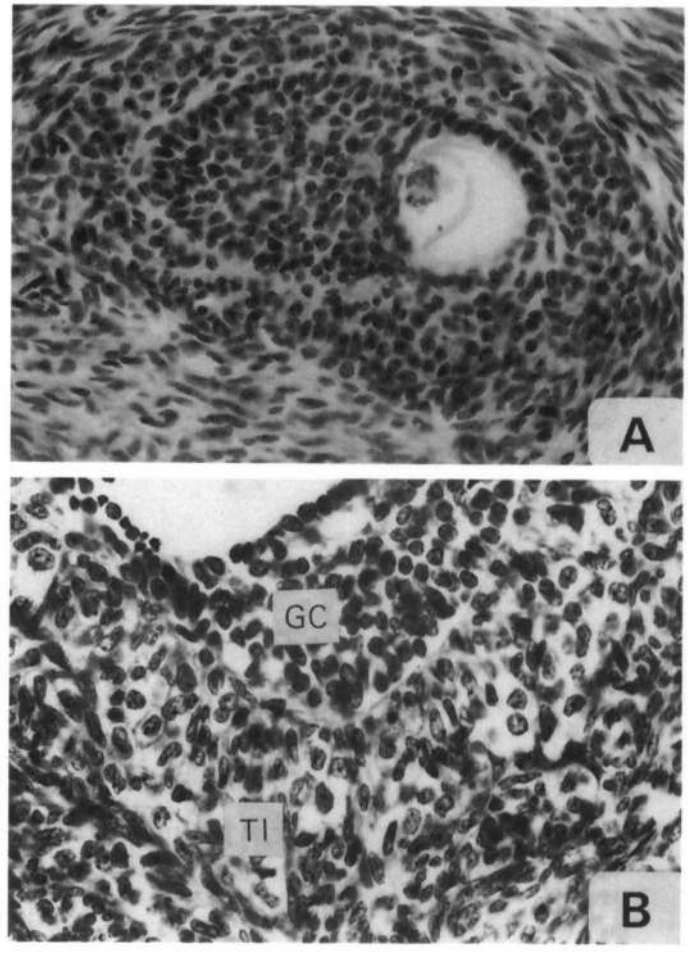

FIG. 9. A, Atretic preantral follicle (×280); the oocyte disappears before the TI hypertrophies. B, Atretic early antral follicle (×480). The oocyte has disappeared, and the incidence of pyknotic GCs is low but TICs are hypertrophied. [A reproduced with permission from A. Gougeon, *The Ovary*, pp. 21–39 (5).]

the population of RF, reflecting a life-long process on which cyclic losses are superimposed (See *Section II*).

In primates, atresia affects early growing follicles only slightly (10). The mean rate of atresia is moderate and does not exhibit substantial cyclic changes for the smallest growing follicles; it is similar for preantral (class 1) and early antral follicles (class 2), 30% and 32%, respectively, and for 0.5- to 0.9-mm (class 3) and 1- to 2-mm follicles (class 4), 15% and 16%, respectively. In contrast, atresia of the largest follicles varies significantly throughout the cycle, being inversely related to circulating FSH levels (105, 110). The highest incidence of atresia is observed during the midluteal phase for both the 2- to 5-mm (class 5) and 6- to 10-mm (class 6) follicle types, approximately 73% and 100%, respectively (61, 105).

C. Alterations of follicular metabolism during atresia

It is generally assumed that atretic follicles possess an intrafollicular androgenic milieu that distinguishes them from healthy follicles (280). However, this must be considered with caution since healthy antral follicles smaller than 8 mm possess low aromatase activity (111, 112, 281) and exhibit a high FF A/E_2 ratio (115–117), grossly similar to that

observed in early atretic follicles of similar sizes (115). On the contrary, healthy follicles larger than 8 mm differ strongly from atretic follicles of similar size in that, for most of them, their FF A/E_2 ratio is between 0.1 and 1.1, and they possess aromatase (112), whereas the A/E_2 ratio in atretic follicles is between 1.4 and more than 40 (115), and aromatase activity is lacking or poorly expressed (112). In addition, binding of FSH to GCs of atretic follicles is patchy and reduced when compared with healthy follicles (282); therefore, as large human follicles degenerate, their GCs appear to lose their capacity to respond to FSH and, rather than being a cause, the absence of aromatase appears to be a consequence of this altered follicular responsiveness to FSH.

Other alterations in follicular metabolism have also been observed in human atretic follicles. They include the appearance of lipid droplets, 3β-HSD, glucose-6-phosphate dehydrogenase, acid phosphatase, and aminopeptidase, as well as a profound decrease in the levels of lactate dehydrogenase in FF of human atretic follicles when compared with healthy follicles (283, 284).

D. Causes of atresia

1. Mechanisms leading to activation of the apoptotic cascade. The generation of oxidative free radicals (oxidative stress) is a consequence of normal cellular respiratory metabolism and reduction-oxidation (redox) reactions (for review see Ref. 285). Exposure of hepatocytes to a redox-active compound that induces oxidative cell injury activates a Ca^{2+}-sensitive endonuclease leading to DNA cleavage preceding loss of cell viability (286). At the ovarian level, it has been shown, first, that GCs collected from ovaries of gonadotropin-primed rats contain high levels of a Ca^{2+}/Mg^{2+}-sensitive endonuclease that can be activated by increased nuclear concentrations of Ca^{2+} and Mg^{2+} (287), and second, that oxidative stress induces GC apoptosis (288). In addition, DNA damage, such as that initiated by oxidative free radicals, may be a primary stimulus for increased p53 (the product of the p53 tumor suppressor gene) expression in GCs (289). Since p53 can increase the transcriptional activity of death-inducer genes, and immunohistochemical studies show that p53 is essentially restricted to nuclei of degenerating GCs of atretic antral follicles, it has been suggested that p53 has the potential to amplify the negative effect of oxidative free radicals on GC viability (Ref. 289 and references therein).

Taken together, these findings indicate that oxidative stress may be a key element in inducing GC apoptosis via alteration of the cellular ionic environment, which activates a Ca^{2+}/Mg^{2+}-sensitive endonuclease resulting in activation of the apoptotic cascade. This, however, poses the question of which factor(s) is (are) susceptible of activating/repressing stress response factors to prevent/induce follicular atresia.

2. Alteration of the tropic hormonal signal. Gonadotropins have been reported to play a critical role in preventing apoptosis in GCs of rat antral follicles *in vivo* and *in vitro* (288, 290). Consequently, insufficient or absent tropic hormonal signal may be pivotal in inducing atresia. This may occur when circulating FSH is absent or too low. Many sources indicate that hypophysectomy leads to the general atresia of all growing follicles from the antral stage onward (for review see Ref. 275), but this is not merely a physiological situation. During the menstrual cycle, circulating levels of FSH exhibit important variations: FSH is highest during the first half of the follicular phase and lowest during the midluteal phase, and during this last phase selectable follicles exhibit their highest rate of atresia.

Recently, Tilly et *al.* (288–291) provided new findings on mechanisms by which insufficient FSH support can induce atresia. By inducing synchronous apoptosis in a homogeneous population of antral follicles by culture in serum-free conditions without tropic support, these authors have shown that the ability of PMSG to inhibit apoptosis was mimicked by inhibitors of oxidative free radical formation and action, including superoxide dismutase, catalase, N-acetyl-L-cysteine, and ascorbic acid (288). These results confirm the role of oxidative stress in inducing atresia, a process that may be prevented by action of oxidative stress response factors. Among them, the product of the *bcl-2* gene acts as an antioxidant or free radical scavenger (Ref. 291 and references therein). It has been hypothesized that the underlying events involved in triggering GC apoptosis and atresia would involve members of *bcl-2* gene family (*bcl-2*, *bax*, *bcl-*x_{long}, and *bcl-*x_{short} genes) (Ref. 291 and references therein). Briefly, the fate of a GC during development may be decided by the ratio or balance of death repressors (*bcl-2* and *bcl-*x_{long}) to death inducers (*bax* and *bcl-*x_{short}), the shift in the *bcl-2* to *bax* ratio being primarily the result of a marked change in *bax* expression with essentially no change in the level of *bcl-2*. Apoptosis induced in follicles incubated *in vitro* without tropic hormones is associated with a marked increase in the amount of *bax* mRNA; conversely, by reducing the amount of both *bax* and *bcl-*x_{short} mRNAs present in GCs, FSH has the ability to prevent apoptosis (291).

Consequently, it is possible that changes in *bax* expression provide a primary signal that determines whether follicles are to survive (low *bax*) or undergo atresia (high *bax*). Thus, it may be that members of the *bcl-2* gene family function as part of a cohort of oxidative stress response factors that must be either induced or repressed to maintain cell survival. In the absence of FSH, steroids, and growth factors (291), the resultant increase in oxidative free radicals may activate Ca^{2+}/Mg^{2+}-sensitive endonuclease that, ultimately, leads to cell death.

Moreover, the Ca^{2+}/Mg^{2+}-sensitive endonuclease, which is inactive in GCs that are not dying (287), seems to be present only in GCs that are sufficiently differentiated, *i.e.* that are responsive to FSH. In rats, immature GCs do not express the Ca^{2+}/Mg^{2+}-sensitive endonuclease, but treatment with PMSG results in a rapid increase in its level in parallel with GC differentiation (287). This observation is in agreement with the view that GC apoptosis is only initiated in follicles that have undergone transition to the antral stage of development (Ref. 288 and references therein) and with the observation that in humans the atretic pathway strongly differs between follicles less than approximately 1 mm (Fig. 9) and follicles larger than 1 mm, where pyknotic GCs are predominant (Fig. 8). Consequently, it is possible that FSH, in addition to inducing differentiation of the GCs, concomitantly increases expression of both the Ca^{2+}/Mg^{2+}-sensitive endonuclease and oxidative stress response factors as a means to

protect the maturing GCs from the damaging effects of metabolically derived reactive oxygen species.

3. Other possible causes of atresia. In addition to an insufficient tropic support, numerous mechanisms or chemical messengers have been reported to regulate GC death in various model systems. Most of them activate endonuclease activity or disrupt prevailing ionic gradients.

The basement membrane must undergo continuous remodeling during folliculogenesis in order for the follicle to be able to expand in size. It is likely that the remodeling process is under hormonal control. Inappropriate hormonal stimuli or specific hormonal signals of atresia may alter the nature of the basement membrane remodeling process, resulting in breaches of the permeability barrier and either exposure of GCs to immune attacks (292) or disruption of the prevailing ionic gradients leading to activation of resident endonuclease (4).

It has also been suggested that atresia may be induced by chemical messengers such as GnRH and angiotensin II (Ang II). GnRH has been reported to be an atretogenic factor in the rat ovary, because a GnRH agonist increases apoptotic DNA fragmentation (293) and inhibits GC mitosis (294). Furthermore, when cultured GCs are exposed to LHRH, there is a rapid and transient increase in intracellular Ca^{2+} (295). In hamsters, Ang II treatment of follicles cultured *in vitro* reduces the E_2/androgen ratio and induces morphological changes in the TI that are consistent with atresia (296), and in cattle, it has been suggested that an inappropriate local production of prorenin, the precursor of Ang II, may play a role in follicular atresia leading to the inhibition of GC aromatase activity (297). In humans, however, GnRH, GnRH receptors, and their mRNA have been detected in/on GCs from preovulatory follicles only (298–300). Similarly, GCs from healthy follicles in normal women immunostain negatively for renin and Ang II until the midcycle gonadotropin surge (301). Taken together, these findings suggest that neither GnRH nor Ang II can act as atretogenic factors in growing nonovulatory human follicles.

In rats, it has been observed that androgens enhance follicular atresia *in vivo* (for review see Ref. 275) and, more recently, that androgens activate endonuclease activity and enhance GC apoptosis (277). However, these experiments must be considered with caution since they have been performed in hypophysectomized, diethylstilbestrol-primed, immature rats, a unique situation in which FSH is absent. In humans, it has been shown that in follicles where A production exceeds GC-aromatizing ability, A is metabolized to dihydrotestosterone (DHT) and 5α-androstanedione, irrespective of the amount of FSH present (110). It has been speculated that DHT and 5α-androstanedione induce atresia by blocking the FSH-stimulated aromatase activity by cultured GCs (281). However, this assumption must be considered cautiously since it has been shown that DHT enhances *in vitro* FSH-induced aromatase activity in GCs from small antral marmoset selectable follicles (302). Taken together, available data indicate that there is no definitive evidence concerning a possible role of androgens in inducing atresia during *in vivo* primate folliculogenesis

In rats, IGFBP-4 has been hypothesized to be involved in the induction of atresia of selectable follicles not selected to become dominant (303). Taking into account the putative role of IGFs in regulating the differentiation and proliferation of GCs and TICs, it may be expected that locally produced IGFBPs can sequester IGFs in the microenvironment and direct nonselected follicles toward atresia. In humans, IGFBP-2 and -4 have been reported to be higher in presumptive atretic follicles (high A/E_2 ratio) than in large (>8 mm) healthy nondominant follicles (low A/E_2 ratio) (170, 304). However, these findings do not necessarily signify that high intrafollicular concentrations of IGFBP-2 and -4 are necessary and sufficient for follicular atresia. High levels of IGFBP-2 and -4 mRNAs are present in healthy 0.5- to 1-mm follicles (162), and IGFBP profiles do not differ between normal follicles smaller than 8 mm with a high A/E_2 ratio, PCOS, and atretic follicles of similar sizes (170–172, 304). We hypothesize that high levels of IGFBP-2 and -4 in large atretic follicles may be a consequence, rather than a cause, of the process by which they have not been selected to ovulate.

In conclusion, although a coherent picture on the mechanisms that may lead to activation of the apoptotic cascade is emerging, little is known of the biochemical and molecular pathways used by various factors to control the initiation of apoptosis. It is possible, however, that they act either by repressing expression of oxidative stress response factors or by favoring expression of death-inducer genes. The early hypothesis (305), that only follicles reaching a critical stage of their development coincidentally with the appropriate changes in serum gonadotropins and/or other factors will be spared from atresia and reach ovulation, remains fully justified in that FSH and certain growth factors have been demonstrated to act indirectly as antioxidative molecules (288).

VII. Synthetic Views on the Control of Follicular Development in Primates

In this section, we will first examine the changes associated with entrance of secondary follicles into the preantral stage and the possible physiological implications in the subsequent steps of follicular growth. Second, we will propose answers to some intriguing questions in relation to folliculogenesis, using data from morphological, immunohistochemical, and biochemical studies that will be integrated and analyzed in the light of *in vitro* studies.

A. Entry of follicles into the preantral class during the early luteal phase: physiological implications

Secondary follicles become preantral when at least one TIC appears to be epithelioid. In humans, follicles enter the preantral stage throughout the menstrual cycle, but it has been observed that it is during the early luteal phase that the number of preantral follicles is highest and their mean size the smallest, reflecting a recent increased entry into this class. In addition, their thecal vascularization is significantly more developed than at any other time during the cycle.

1. Which factors are involved in the increased vascularization of preantral follicles? Angiogenesis is a complex developmental process that undoubtedly results from multiple interactions

(for review see Ref. 217). A role for LH has been suggested since in humans, hCG increases the influx of blood within follicles (67). The angiogenic effect of LH may be exerted via stimulation of Ang II production by preovulatory GCs (306) that, in turn, may stimulate bFGF secretion (307), one of the most potent angiogenic factors (217) to be detected in the human ovary (218–220). In addition, FF from follicles sampled during the follicular phase in unstimulated menstrual cycles possess a mitogenic potential for bovine aortic endothelial cells (308), and IVF-GCs can synthesize an endotheliotropic factor *in vitro* (309). Messenger RNA coding for vascular endothelial growth factor has been identified only in GCs of antral and preovulatory monkey follicles (310). This protein induces angiogenesis under *in vivo* conditions and has been suggested to be stimulated by FSH (6, 217). It is also important to note that many substances involved in follicular rupture and produced by the ovulatory follicle after the gonadotropin surge are angiogenic factors as well (for review see Ref. 217). Other angiogenic factors can be produced by the preantral follicle itself. This is the case for TGFα, which stimulates endothelial cell proliferation in hamsters (311) and has been detected at the theca level and in GCs as early as the primary stage in humans (51).

Available data, therefore, indicate that angiogenic factors may originate from follicles of various sizes, from the secondary to the preovulatory stage. Although the mode of action of these peptides remains unclear, they may act locally or via the general circulation to enhance thecal vascularization of preantral follicles during the periovulatory period.

2. *Is there a coincidence between the periovulatory period and the differentiation of TICs?* Although secondary follicles can transform into preantral follicles throughout the menstrual cycle by acquisition of epithelioid cells in the theca layer (*i.e.* differentiation of TICs), there is a significant increase in the number of preantral follicles during the early luteal phase. LH has been considered to be the primary hormone regulating the differentiation of TICs (312). However, some observations in rats suggest that a regulatory factor distinct from LH stimulates thecal differentiation and that IGF-I, in stimulating the initial expression of LH-R during the early stages of thecal differentiation, may be this factor (313). In addition, observation of atretic preantral follicles shows that TICs hypertrophy soon after disappearance of the oocyte in humans (Fig. 9) as well as in rats where these cells develop the ultrastructural and functional features characteristic of steroidogenic tissues (314). These findings suggest that in healthy follicles a factor may exert an inhibitory influence on theca cells to prevent their differentiation. Potential candidates are EGF and TGFα, which have been detected in human oocytes and GCs, respectively (50–52), and have been postulated (208) to function as theca-organizing factors that attract theca cells around the follicle while maintaining them in an undifferentiated stage. Thus, differentiation of the TI that occurs during the early luteal phase in a higher number of preantral follicles than at any other time during the menstrual cycle may result from an increasing amount of extraovarian IGF-I reaching secondary follicles via increasing vascularization. By its ability to induce differentiation of protheca cells, IGF-I may counteract, at least partly, the inhibitory influence exerted by GCs and/or the oocyte on this differentiation.

3. *Do changes represent protective mechanisms of a particular wave of follicular growth?* In the mouse, it has been shown (315) that the passage of a follicle toward atresia or ovulation is determined by the time of the cycle when follicles reach stage 5a (similar to the human preantral stage). Moreover, Greenwald and Terranova (316) suggested that hamster follicles developing in synchrony with FSH and LH surges recurring every 4 days constitute the group selected to ovulate after four or five cycles of development.

In both hamsters (317) and rats (318), FSH stimulates follicular production of EGF and EGF-R, and in rats, it has been shown that GC apoptosis is prevented by a tonic inhibition of endonuclease activity by the paracrine/autocrine action of EGF/TGFα (290). Because TGFα and EGF are present in both TICs and GCs in preantral and early antral human follicles, respectively (Table 7), it is possible that follicles that have differentiated their TI in an optimal gonadotropic environment, *e.g.* during the periovulatory period, possess the ability to synthesize sufficient amounts of EGF/TGFα to be better protected against atresia than follicles that have differentiated their TI at other times during the cycle. These latter follicles may constitute the "minor" waves that normally do not lead to a preovulatory follicle; this hypothesis is in agreement with the early Richards' hypothesis (126) of an "inadequate" differentiation of the TI resulting in an imbalance detrimental to follicular development in subsequent stages of growth.

From these findings, we speculate that, in women, follicles differentiating TI during the periovulatory period might be endowed with selective advantages for further normal development by periovulatory gonadotropin changes acting via increased blood vascularization. These follicles may constitute the "major" wave from which follicles will become selectable 70 days later. Nevertheless, whether all or only some members of the major wave are equally privileged (endowed with selective advantage) to become selectable is not known and requires further study. The existence of such waves could also explain why the passage of follicles from one class to the next is detected only once per cycle (see *Section III.C*). Inasmuch as they are less susceptible to atresia than follicles from minor waves, follicles from major waves will be found in larger numbers throughout the successive cycles, which explains why their passage through subsequent classes is discernible (Fig. 10).

B. Basal follicular growth

In the human ovary, the number of follicles with a small antral cavity peaks during the late follicular phase. Because the primary stimulus for antral transition is believed to be FSH (4), and because FSH alone is sufficient to induce antrum formation in human preantral follicles cultured *in vitro* (108), this increased entry of follicles into the early antral class may be related to the high levels of FSH observed during the first half of the follicular phase. In addition, it has been shown in rats that antrum formation requires the presence of the oocyte and that the action of FSH is enhanced by activin A (237). However, the precise mechanisms by which activin and the oocyte may be involved in antral transition remain unknown.

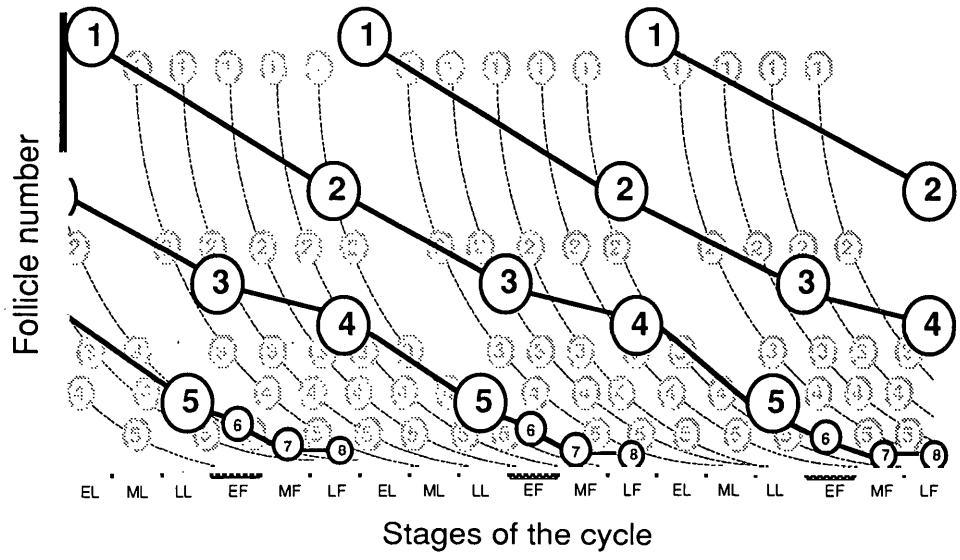

FIG. 10. Description of the progression of the "major wave" throughout successive cycles, from the entry of follicles into the preantral (class 1) to the preovulatory stage (class 8). Follicles that became preantral during the early luteal phase of every menstrual cycle have been endowed with selective advantages for further development. As growth is progressing, the initial number of follicles belonging to the major wave (*bold line*) decreases by atresia, but in lesser proportion than do follicles from "minor waves" (*dotted thin line*). Consequently, when follicles belonging to major waves and of a given stage of their development (*e.g.* class 1) enter the subsequent class (*e.g.* class 2 during the late follicular phase) they are in larger numbers than at any other time during the cycle, thereby explaining why this passage is morphologically discernible.

1. How do follicles develop during basal growth despite their low responsiveness to FSH? We have seen above (*Section IV*) that follicles undergoing basal growth are relatively insensitive to cyclic gonadotropin changes in terms of growth rate and steroidogenesis. In rat GCs, low and constant doses of FSH increase FSH-R mRNA levels, whereas a single high dose of either FSH or LH/hCG decreases FSH binding and FSH mRNA levels (319). Because it has been observed that FSH fails to induce desensitization of adenylate cyclase in GCs of preantral rat follicles (130), it can be assumed that this property of immature GCs may be required to permit a continuous nondisruptive pattern of follicular growth when small follicles are repeatedly exposed to gonadotropin surges.

To explain why follicles can grow during basal growth despite this low responsiveness to FSH, a role for oncogenes and growth factors can be suggested. The c-*myc* mRNA is abundantly expressed in GCs from 1- to 2-mm as compared with 3- to 11-mm porcine follicles (320), and only GCs from preantral follicles immunostain positively for the *myc* protein in humans (55). Evidence suggests that c-*myc* oncogene expression is linked to growth control in the prereplicative phase and, as a result, the intriguing possibility of c-*myc* oncogene participation in the "autonomous" growth of the immature follicles during the early stage of folliculogenesis has been proposed (320). In addition, EGF/TGFα and IGFs have well known effects on human GC proliferation (Tables 5 and 7). Because EGF/TGFα and their receptor, as well as IGF-R, have been detected in preantral and small antral human follicles, it is likely that these factors may play a positive role in sustaining growth of small follicles.

2. Why is steroidogenic activity low in follicles during basal growth? The steroidogenic activity of follicles during basal growth is very low. A major cause for this appears to be the lack of steroidogenic enzymes in TICs ($P450_{scc}$ and $P450_{17\alpha}$) and GCs ($P450_{arom}$). When the physiological effects of EGF/TGFα (Table 6) are integrated with their developmentally regulated production, it appears that these peptides have the ability to block follicular steroidogenesis during basal growth, by inhibiting the FSH-induced aromatase activity in GCs and the LH-induced androgen production in TICs. The underlying mechanism of inhibition by EGF of the FSH-induced aromatization is not fully understood, but it has been reported that EGF reduces cAMP formation by reducing adenylate cyclase activity (321). Similarly, in TICs, EGF inhibits cAMP-stimulated $P450_{17\alpha}$ activity (150). TNFα, immunohistochemically localized in small healthy antral follicles of the human ovary (265), may also be involved in the inhibition of steroidogenesis, because of an existing dose-dependent inhibitory effect of TNFα on basal or LH-stimulated A production by TICs from immature rats (322). In addition, it has been suggested in rats (323) that TNFα may be an important modulator of the gonadotropin-induced transition of GCs from a proliferative to a differentiative state. Although the cellular mechanism of this regulation is yet to be determined, it is possible that the mitogenic influence of this cytokine, and maybe of EGF/TGFα, is dominant over the differentiated function of FSH and, as such, promotes the progression of the GCs from the G1-G0 to S phase of the cell cycle and results in the "switching off" of FSH-induced differentiation.

C. Selectable follicles

Selectable 2- to 5-mm follicles are present throughout the cycle. In contrast to smaller follicles they are sensitive to cyclic changes in FSH since both the percentage of healthy follicles and mitotic index of their GCs are low when FSH is low and are high when FSH is high. In addition, the hMG-induced GC proliferation is highest when women are treated

during the late luteal phase (114). However, although their TICs synthesize significant amounts of aromatizable androgens, aromatase activity of their GCs and subsequent E_2 production (Table 3) remain very low.

1. How do these follicles respond to changing levels of FSH by altering GC proliferation? Both activin and EGF, present in primate selectable follicles, stimulate IVF-GCs (200, 229) and/or immature GCs (199) to proliferate. The highest levels of inhibin β_B-subunits have been observed in selectable monkey follicles (245). Therefore, because treatment of GCs with FSH in rats enhances the number of activin-R (324), and *in vivo* treatment with FSH increases the ovarian EGF-R content approximately 2-fold (318), it is possible that activin, acting alone, and EGF, acting in synergy with FSH, stimulate proliferation of GCs in human selectable follicles when FSH increases. Follicular vascularization may also be involved in this process. In rabbits, it has been observed that the number of thecal capillaries increases linearly with increasing follicular size (325), thereby increasing the blood flow rate and consequently the amount of both FSH and IGF-I reaching the follicle. Consequently, it appears likely that in human selectable follicles, activin, EGF, and IGF-I, acting alone or synergistically (326), have the potential to increase the FSH-induced GC proliferation when FSH levels increase, especially during the late luteal phase.

2. Why does production of E_2 remain low in selectable follicles? The mean concentration of A in FF from selectable follicles is of 638 ± 113 ng/ml whereas that of E_2 is 15 ± 5 ng/ml (Table 3). TICs of these follicles do not stain positively for $P450_{scc}$ and $P450_{17\alpha}$, suggesting that these enzymes are present in low quantities. However, these cells possess IGF-R (Table 5) and more LH-R than those of follicles during basal growth (123, 124). Consequently, extraovarian IGF-I and LH, which have positive effects on androgen production by TICs (89, 151), may, via increasing theca blood vascularization, stimulate biosynthesis of A and counteract the negative effects of both activin (149) and EGF (150, 208) on androgen production by TICs.

Although both FSH and IGFs stimulate *in vitro* production of E_2 by GCs independently of cell maturity (Table 4), and despite the presence of IGF-R on GCs (Table 5), these follicles synthesize very low amounts of E_2. Consequently, factor(s) inhibiting the aromatization of androgens by GCs may be operating inside the follicle. This is the case for EGF and TGFα, which are present in selectable follicles (Table 7). In addition, IGFBPs, and especially IGFBP-2 and -4 (49), may inhibit the positive action of intrafollicular IGF-II on E_2 production (148). It can be suggested, therefore, that EGF/TGFα and IGFBPs have additive effects that inhibit FSH-induced aromatase activity at the GC level.

Alternatively, it is possible that the "FSH threshold" is lower for inducing GC proliferation than for inducing cytodifferentiation. Continuous *in vivo* thymidine infusion to rats indicated that within each antral follicle the granulosa layer is composed of two distinct subpopulations: proliferating cells and nonproliferating cells undergoing differentiation (327). Thus, it is possible that because of their prevailing proliferative status, GCs in selectable follicles exhibit a very high stimulation threshold for aromatase expression, which may require either a high dose of a single factor or a combination of effects from multiple factors to activate the gene responsible for synthesis of aromatase protein.

D. Selection of the follicle destined for ovulation

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 Hodgen (75) to indicate the final adjustment of the cohort to a size equal to the species-characteristic ovulatory quota. At the beginning of the follicular phase the largest healthy follicle appears to be the selected follicle. It differs substantially from selectable follicles in the A/E_2 ratio in FF that is comprised between 0.1 and 1 (Table 3). Thus, one of the most important events that has occurred during selection is the appearance of aromatase activity in GCs.

1. What regulates changes in steroidogenic activities in the newly selected follicle? In rats, it has been observed that high concentrations of FSH inhibit IGFBP-4 production and increase IGFBP-4 protease activity that hydrolyzes IGFBP-4 into smaller fragments that do not bind the IGF ligands (328). In humans, both IGFBP-2 and -4, which were at high levels in small follicles (49), strongly decrease in preovulatory follicles (171). In addition, GCs from preovulatory follicles produce IGF-II (164) and contain mRNA for IGF-II at significant levels (159). As IGFs are known to act in synergy with FSH to enhance the FSH-induced aromatase activity (Table 4), it can be proposed that in the newly selected follicle free IGF-II may stimulate aromatase activity in synergy with high circulating levels of FSH characterizing the intercycle period. In addition, although the ability of activin in enhancing FSH-induced aromatase activity in GCs is disputed (231), the observation that activin stimulates aromatase in both immature and preovulatory monkey follicles (227) suggests that this peptide may reinforce the action of IGF-II at the time of selection. At the TI level, the negative effect of activin on production of aromatizable androgens may be counteracted, at least partly, by both a higher number of LH-R on TICs of selected follicles (123) and an increasing LH pulse frequency during the early follicular phase (329).

2. How is the follicle destined to ovulate "chosen"? Are these follicles different because they are derived from a different clonal pool? It has been suggested that the selected follicle is the one that is growing most rapidly in response to the intercycle rise in FSH and has differentiating GCs: *i.e.* the one with the lowest FSH "threshold" (61). As demonstrated in the marmoset monkey, GC aromatase activity in preovulatory follicles is at least 10 times more sensitive to FSH than in small follicles (302). The increased responsiveness of GCs to FSH in terms of aromatase activity may be related either to the existence of different transducers to which the FSH-R can couple or to changes in activity of the adenylyl cyclase enzyme through the turning on/off of expression of specific genes (122). Interfollicular differences in ability of these specific genes to be activated or repressed by various molecules, including growth factors, may be involved in the process by

which one follicle, being more receptive than others to FSH, is "chosen" and begins its preovulatory maturation.

The character (stochastic *vs.* deterministic) of these mechanisms remains unknown. That the population of follicular cells (GCs and TICs) present in a given follicle constitutes a mixture of clones that have evolved from different stem cells (330) is probably of great importance. The average number of clonal progenitors has been estimated to be five in mouse GCs (331) and corresponds closely to the numbers of observed pre-GCs surrounding the oocyte in primordial follicles. Consequently, in a follicle that has begun its growth at a given time, the population of GCs is probably different from that of another follicle that started its growth at the same time. GCs of follicles presenting the same morphological characteristics may display differences in their development potentialities and capacities for synthesizing molecules that affect their own growth and differentiation. It is therefore tempting to speculate on the existence, within a population of healthy follicles of similar size, of some follicles presenting functional differences, even if moderate, that lead to a prompter expression of genes governing the synthesis of factors or enzymes favoring growth and ultimately full differentiation. This paradigm may explain why, after several weeks of growth, a given follicle rather than another is selected for ovulation. Being capable of appreciating even minimal differences in the expression of genes controlling the synthesis of molecules involved in follicular development will probably help answer this question.

E. Preovulatory maturation

Between selection and the time of the plasma E_2 peak, the mean steroid concentration in preovulatory FF increases 2-fold. Interestingly, preovulatory FF before the midcycle gonadotropin surge exhibits significant levels of progestins (Table 3). Because GCs from human follicles before the midcycle gonadotropin surge lack 3βHSD *in vivo* (113, 121), progestins observed in FF are probably of thecal origin. This may be due to an effect of TGFβ, whose production is highest in preovulatory TICs (Table 8), and has been reported to enhance forskolin-stimulated 3βHSD activity by cultured TICs from human preovulatory follicles (150).

1. What is the role of the inhibin-activin-FSP system during preovulatory maturation? Studies of FF levels of androgen and estrogen in relation to GC aromatase activity indicate that the capacity of the TI to generate A increases in parallel with aromatase activity in the human preovulatory follicle. It has been suggested that a positive feedback loop (GCs on TICs) promotes thecal androgen synthesis and hence estrogen synthesis in this follicle (94).

The kinetics of production of inhibin subunits in monkey follicles during their growth indicates that the activin gradient of the developing follicle declines as its inhibin gradient increases (245). In addition, throughout preovulatory maturation, FF contains increasing levels (332, 333) of FSP, whose production is under the control of FSH (334). FSP, which preferentially binds activin (242, 335), inhibits its biological effects (235). While the inhibitory effect of activin on production of aromatizable androgens by TIC is progressively raised, preovulatory GCs produce increasing amounts of inhibin (247, 336), which strongly enhances the LH- and IGF-stimulated androgen production by TICs (228) and suppresses the inhibitory action of activin on androgen synthesis by these cells (149, 227). Regarding the FSP action, it is tempting to postulate that this molecule may serve to inhibit or modulate the action of a substance whose synthesis cannot be blocked specifically. During preovulatory maturation, increasing amounts of inhibin are being synthesized, a phenomenon dependent upon the synthesis of α- and β-subunits. Therefore, any substance specifically blocking activin synthesis, whose inhibiting effects on TI androgen synthesis could hamper the development of the follicle destined to ovulate, will also block that of inhibin, thereby confusing the overall effects of the blocking substance. By binding more specifically to activin than inhibin, FSP can strongly inhibit activin action without substantially affecting that of inhibin.

These observations show that, as the preovulatory follicle matures, it produces increasing amounts of inhibin and FSP, which have a positive effect on androgen synthesis by TICs, either directly or indirectly.

The biological effects of inhibin and activin on follicle cells and their changing expression during follicle growth indicate that they may play a key role in folliculogenesis. Studies by Matzuk *et al.* (236), who used inhibin-deficient mice, indicated that in the absence of inhibin, mice can develop into young adults that are not completely normal. Within a few weeks, highly invasive and hemorrhagic tumors that resemble GC tumors develop in the ovary. Whether this abnormal ovarian cell proliferation is due to lack of inhibin or to hypersecretion of FSH is not known. In addition, these animals are infertile. The reason for infertility in these inhibin-deficient mice is not yet understood; the authors suggested that it is secondary to the early development of the tumors either due to hypersecretion of FSH or to the absence of inhibin (236). Although development of normal secondary sexual organs and mature-appearing gametes have been reported, a review of the accompanying microphotographs shows that the ovary from a 4-week old inhibin-deficient animal does not resemble the ovary of a normal animal of the same age in that it contains no antral follicles. Therefore, it is possible that infertility is due not only to the occurrence of tumors but also to an impaired follicular growth.

2. Why does aromatase activity increase before the midcycle gonadotropin surge? FSH is the primary stimulant of functional GC differentiation at the onset of the preovulatory phase (337). At the level of GCs, many substances have been reported to cooperate with FSH to stimulate aromatase expression. T and DHT enhance *in vitro* FSH-induced aromatase activity in GCs from small antral marmoset selectable follicles (302). They may act via androgen receptors present in human follicle cells (338, 339). In rats, E_2 stimulates aromatization of androgens in GCs (126). Although estrogen receptors have been detected in large antral follicles of primates during the follicular phase (338–342), no positive effect of E_2 on FSH-induced aromatase activity has yet been reported in primate GCs. In primates, estrogens probably do not play a key role in folliculogenesis as assessed by the presence of ovulatory follicles after treatment with exoge-

nous gonadotropins in patients suffering from a 17α-hydroxylase deficiency (343) and by the presence of follicular growth after treatment with recombinant FSH in LH-deficient monkeys despite production of E_2 being strongly impaired (84). During preovulatory maturation, production of IGF-II and its mRNA by GCs increases; FF concentration of IGF-II increases (Table 5), while those of IGFBP-2 and -4 strongly decrease. It can therefore be assumed that IGF-II may be the most effective stimulator of GC aromatase activity in synergy with FSH which is also involved in stimulating the expression of GC IGF-R (3). Taken together, these findings indicate that as the preovulatory follicle matures, it produces increasing levels of molecules having a positive effect on production of E_2 by GCs.

3. Why does GC proliferation decrease before the gonadotropin surge? Is this change due to GC differentiation? When the preovulatory follicle begins to produce high levels of E_2, the rate of cell proliferation in the membrana granulosa begins to decline (66). This change, which parallels an increase in the capacity of GCs to produce E_2, *i.e.* to differentiate, can be explained by the progressive change in the ratio of proliferating GCs to nonproliferating GCs undergoing differentiation. Thus, in the newly selected follicle some GCs differentiate, as compared with selectable follicles, and begin to produce E_2 while FSH, acting in synergy with factors favoring proliferation, stongly stimulates the growth of a large number of remaining proliferative GCs. The more the follicle matures, the less there are GCs that retain their capacity to proliferate. FSH, acting in synergy with cytodifferentiative factors, causes more GCs to differentiate and express the FSH-induced functions resulting in a simultaneous decrease of the GC proliferation and increase in E_2 production. At this time, adenylyl cyclase, which is highly responsive to FSH (130), produces high levels of cAMP, which has been shown to inhibit GC proliferation in cattle (344), possibly by acting on cell-cycle factors.

4. How does the preovulatory follicle exert its dominance upon less developed follicles? The follicle destined to ovulate has been termed the "dominant follicle" (345), a concept originating from the observation of a bilateral arrest in the development of the less mature follicles in the presence of the preovulatory follicle. Thus, destruction of the dominant follicle during the late follicular phase is followed by a gonadotropin surge occurring 2 weeks later, *i.e.* the time interval necessary for a selectable follicle to reach the preovulatory size, in both monkeys (75) and women (76). This delay corresponds with the situation prevailing in humans, where the mean diameter of the largest nonovulatory healthy follicle is 2.4 ± 0.7 mm during the late follicular phase (60). In addition, it has been shown that the hMG-induced GC proliferation in selectable follicles decreases as the time of ovulation approaches (114) and that the hMG-induced follicular growth is suppressed in the presence of the preovulatory follicle (346). These data confirm that in the presence of a preovulatory follicle, the mechanisms leading a follicle to be selected are inhibited. DiZerega *et al.* (for review see Ref. 347) assumed that dominance originates from the secretion of follicular substance(s) termed "follicular regulatory protein(s)," which have the ability to suppress the follicular response of less developed follicles to gonadotropins by inhibiting their gonadotropin-stimulated aromatase. To date, the exact nature of follicular regulatory protein has not been elucidated. This hypothesis of a direct inhibition of less-developed follicles by the preovulatory follicle has been disputed by others who suggested an indirect dominance via increasing circulating levels of E_2 (348, 349) and subsequent fall in FSH occurring from the midfollicular phase.

5. How does the preovulatory follicle continue to thrive when FSH levels are decreasing? Although FSH appears to be essential for the final growth of the follicle destined to ovulate (349), its GCs bind less FSH than do those of early preovulatory follicles in midfollicular phase (124, 282), and from the midfollicular phase onward, the circulating levels of FSH progressively fall in response to increasing plasma E_2 levels.

Despite less circulating FSH, the amount of FSH reaching the dominant follicle may be either unchanged or increased since it has been shown (69) that during the second half of the follicular phase, the preovulatory follicle is much more vascularized than are less developed follicles. Production by the preovulatory follicle of angiogenic factors, including vascular endothelial growth factor (310), bFGF (219, 220), Ang II (306), cytokines (217), or unidentified factors (309, 350), may account for the increase in thecal vascularization. Thus, a causal relationship between gonadotropin stimulation and the expression of angiogenic substances suggests an intraovarian mechanism by which the dominant follicle protects itself from the fall in plasma FSH concentrations. That is, by developing an elaborate vascularization network, the dominant follicle may preferentially increase its exposure to gonadotropins. Moreover, a change in FSH-R function, or peptides actively synthesized by the dominant follicle itself, may play a basic protective role by enhancing sensitivity of GCs to FSH. It has been demonstrated in the marmoset monkey that GC aromatase activity and P accumulation are at least 10 times more sensitive to FSH in preovulatory follicles than in small follicles (302, 349), and in rats, GCs isolated from preovulatory follicles exhibit greater FSH-responsive adenylyl cyclase than GCs isolated from small antral follicles in which steroidogenesis is low (130). It has also been suggested that the presence of LH-R on preovulatory GCs, which appear during the midfollicular phase (124, 127) under the primary control of FSH acting in synergy with E_2 (126), TGFβ (222), and IGFs (351), could serve to protect the follicle from the fall in plasma FSH concentrations by allowing the cells to produce cAMP. Thus, an increase in angiogenesis and sensitivity of GCs to LH and FSH may explain the maintenance of the preovulatory follicle development during the late follicular phase when circulating levels of FSH are decreasing.

F. Preovulatory growth after the midcycle gonadotropin surge

After the midcycle gonadotropin surge, the preovulatory follicle switches from a E_2-producing to a progestin-producing structure. Its production of steroids strongly increases, rising from a mean FF concentration of near 4,800 ng/ml

before the surge, to nearly 11,000 ng/ml after (Table 3). In addition, GC proliferation is arrested and capillaries from the theca layers invade the granulosa.

1. What controls the shift in preovulatory steroidogenesis after the gonadotropin surge? Although FSH and growth factors, such as IGFs (141, 144, 147), and FSP (235) in humans and TGFβ (352) in rats, have been demonstrated to stimulate progestin production, LH is primarily involved in the shift of steroidogenesis observed after the midcycle gonadotropin surge. The decrease in intrafollicular E_2 and A after the midcycle gonadotropin surge could be due to an LH-induced inhibition of the thecal $P450_{17\alpha/lyase}$ complex since direct measurements on human GCs during the gonadotropin surge (353) or just before ovulation (354) have shown that aromatase activity remains high. Moreover, GCs from preovulatory follicles in marmoset monkeys express an LH-responsive aromatase system (355). It is evident that $P450_{17\alpha/lyase}$ is not completely inhibited because both 17α-OHP and E_2 remain present in FF at significant levels (115). The decreased secretion of androgen and estrogen observed after the LH surge may be, at least in part, accounted for by a decrease in mRNAs for $P450_{17\alpha}$ and/or $P450_{arom}$ in both bovine (356) and rat (357) GCs. However, it is important to note that the decline in aromatase mRNA in the differentiated GCs is not necessarily associated with a corresponding loss of aromatase activity, the half-time of the enzyme being much longer than that of its mRNA (357). Alternatively, an alteration of the 17βHSD activity may be involved because data in rats suggest that the ovulatory surge of LH can down-regulate the expression of this enzyme (358).

Concomitantly to the decrease in E_2 and A production in GCs after the gonadotropin surge, P production increases markedly (Table 3). This results from two additive effects. First, both 3βHSD (66, 113, 121) and $P450_{scc}$ (111) appear in GCs after the gonadotropin surge. Second, 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 (for review see Ref. 64).

2. Which mechanism arrests mitoses in GCs after the gonadotropin surge? In addition to the possibility that after the LH surge GCs having completed the final step of their maturation become incapable of proliferating, a direct action of P to block GC mitosis has been suggested. At concentrations similar to preovulatory intrafollicular levels, P directly inhibits the proliferation of cultured IVF-GCs, an effect not mimicked by E_2, T, DHT, or dexamethasone, and which cannot be overridden by EGF (359). Aminoglutethamide, which blocks P secretion, increases the number of cultured IVF-GCs (359). The growth-inhibiting effect of medium containing high levels of P is removed by either RU 486 or charcoal extraction, but not by heat inactivation (360). After the gonadotropin surge, primate GCs possess P receptors (340, 361–363), so it appears likely that P plays a role in inhibiting GC proliferation.

VIII. Conclusions and Perspectives

A simultaneous analysis of morphological and functional changes of follicles during their development has allowed the construction of a coherent picture concerning the mechanisms, sometimes redundant, that may control ovarian folliculogenesis in primates. Yet, this reviewer is fully aware that many pieces of this puzzle remain schematic and speculative for several reasons.

First, only peptides either present in primate follicles or having an effect on primate follicle cells have been considered in the current review. This does not signify that other peptides present in large number in the mammalian ovary are not involved in regulation of folliculogenesis, and their possible role requires further studies.

Second, the cellular signaling pathways in follicle cells and the hormonal control of gene expression in the ovary are highly complex and have not been integrated in our model. A better understanding of follicular growth needs to take into account these mechanisms (for review see Ref. 364).

Finally, we must wonder about the exact role played by growth factors in the regulation of ovarian folliculogenesis. One of the most fascinating features of the intraovarian regulatory network controlling follicular growth is the redundance of systems that assure the proper function of the ovary, *i.e.* the production of one fertilizable oocyte every cycle. For both the control of follicular development and destruction of excess follicles through atresia, there is probably more than one single triggering factor. The molecules involved in keeping the follicular tissue in an undifferentiated state, such as those responsible for the acquisition of gonadotropin responsiveness by these same tissues, never act alone. In each step there are several actors involved. As a result, a deficiency in any one of the players controlling one or several functions may be compensated for by the action of other substances. Nevertheless, despite many *in vitro* experiments arguing in favor of a major role played by intraovarian factors in the regulation of folliculogenesis, a significant doubt persists as to the true physiological role they play *in vivo*. Because all these substances are ubiquitous and are involved in the functioning of numerous other tissues, any attempts at clarifying their function through their injection or neutralization through antisera are rendered very difficult, if not illusory. By using targeted deletion of gene (knock-out studies) governing production of various growth factors [inhibin α-subunit (365), activins (366), activins and activin receptor type II (367), FSP (368), TGFβ1 (53)], it has been possible to analyze the effect of these factors on embryonic development and reproductive function. However, one problem raised by transgenic animals is that of their viability. For example, although activin-βA-deficient mice progress to term at the expected frequency, they died within 24 h of birth, eliminating the possibility of analysis activin effect on cyclic folliculogenesis (366). Another problem is linked to the action of peptides of the inhibin family at both the pituitary and ovarian levels. Thus, while in inhibin-deficient mice, the circulating levels of FSH are markedly increased (365), they are strongly decreased in activin-deficient mice (367). Consequently, it is likely that impaired ovarian function is due to abnormalities in circulating levels of FSH. However, it

appears impossible to conclude whether absence of the growth factor at the ovarian level is responsible, at least in part, for the observed defects. It appears, therefore, that the use of knock-out transgenic mice to improve our knowledge of growth factor effects in ovarian function is limited, since these peptides are ubiquitous factors and are involved in the functioning of numerous other organs.

Alternatively, the use of two other transgenic mice models may be of greater interest. In animals bearing a dominant-negative mutant protein, the overexpression of the mutant protein can be induced by the use of a tissue-specific promoter-enhancer element leading to an experimental deprivation of the normal protein (369). In animals bearing a human TGFα DNA under the control of metallothionein-1 promoter, the TGFα gene can be either acutely or chronically overexpressed (370). However, because there are differences among species (rat, 371; and human, 51,214) with respect to the presence and function of TGFα in the ovary, this latter study raises the problem of specificity of action of growth factors. This specificity dictates that one must be cautious in extrapolating the results obtained in rodents to primates.

Nevertheless, despite these various consequences, transgenic mice models constitute promising tools with which to study further the various autocrine and paracrine systems that may play a basic role in regulation of follicular growth.

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