PART I

PHYSIOLOGY OF REPRODUCTION

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FOLLICULOGENESIS: FROM PREANTRAL FOLLICLES TO CORPUS LUTEUM REGRESSION

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INTRODUCTION

The most common function of the female gonad is to produce gametes, the oocytes, and sex hormones, such as estrogens and progesterone, which control the development of the female secondary sexual characteristics and support pregnancy. These two functions are exerted cyclically between puberty and the menopause, and they are regulated by diverse endocrine and paracrine factors acting on many cell types situated in the ovary. Ovarian functions result from the evolution of a morphological unit, the ovarian follicle, which consists of a central oocyte surrounded by granulosa cells and other layers of somatic theca cells (1). The maturation of the follicle proceeds through primordial, primary, and secondary stages of development and is controlled by various factors produced in the ovary. The main physiological stimulants for differentiation and luteinization of granulosa cells, which are a main cellular component of the follicle, are the gonadotropin hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Throughout the reproductive life span of the female, only limited number of follicles will reach the stage of Graafian follicle and will ovulate, whereas the vast majority is gradually eliminated through a process called atresia. In every menstrual cycle, only one follicle, named the dominant follicle, is destined to complete maturation and ovulate, and thus, the formation of the multiple embryos during pregnancy is prevented.

Degeneration of the old corpus luteum is a process essential for maintaining the normal production of progesterone in every menstrual cycle. The complexity of the interrelation of the events that control oocyte growth and ultimate acquisition of developmental competence is under continuous investigation (2). It is generally believed that follicular atresia and luteolysis occur by mechanisms that accompany a highly organized type of cell death, called programmed cell death or apoptosis (3). The present review reports a variety of factors involved in the different stages of follicular development. Elucidation of the mechanisms that regulate follicular development may lead to the prevention of female reproductive disorders or other pathological conditions and to the development of new culture methods for oocytes for in vitro fertilization.

PREOVULATORY FOLLICLE

The development of preantral follicles involves oocyte enlargement, zona pellucida formation, extensive granulosa cell proliferation, formation of a basal lamina, condensation of stromal cells around the basal lamina to form the theca layer, and the development of fluid-filled spaces that gradually coalesce to form the antral cavity (4, 5). In the absence of appropriate gonadotropic stimulation, follicles develop until the early antral stage and atresia occurs.

The early stages of follicular development, including the early antral follicle, are independent of the FSH and the luteinizing hormone (LH). In agreement with these findings is the study of Touraine et al., which shows that inactivation of FSH receptor does not disrupt the follicular growth to the large preantral stages (6). The low responsiveness of antral follicles to gonadotropins results presumably by the low number of gonadotropin receptors on follicle cells at this stage of development, although it is believed that anti-Mullerian hormone (AMH) reduces the FSH responsiveness of preantral and small antral follicles (7). However, the preantral follicle is affected by other nongonadotropic factors, such as members of the TGF-β family, estrogens, androgens, insulin, and insulin-like growth factor-1 (6, 1). The follicles at the preantral stage are shown to produce very low amounts of progesterone, and androstenedione and no estradiol production is detectable (8) and possesses only faint aromatizing capacity.

A Graafian or antral follicle measures 0.4–2.3 mm in diameter and is characterized by a cavity of antrum containing a fluid termed follicular fluid. The development of the antral cavity begins with the formation of a cavity on the one pole of the oocyte. After antrum formation, the development of the follicle precipitates, and in sixty days, the follicle reaches the preovulatory stage. The size of an antral follicle is mainly determined by the size of the antral cavity and the proliferation rate of the follicle cells. In a dominant follicle, for example, extremely rapid proliferation of granulosa and theca cells occurs, and therefore, the dominant follicle is correlatively bigger in size than any other follicle during the follicular phase of the cycle.

During antrum development, the follicles acquire capillary networks, located in the theca interna and externa. The blood vessels increase in number and size as follicular development proceeds but do not penetrate the basal membrane (9). It is believed that VEGF, a mitogenic factor, is involved in angiogenesis process and thus in antral cavity formation, through VEGF receptors. It has been shown that inhibition of VEGF results in decreased follicle angiogenesis, reduced recruitment, and growth of antral follicles in the primate. VEGF is also

thought to be involved in the ovulatory process, as other studies correlate the VEGF with local factors involved in ovulation (10). Furthermore, suppression of VEGF in the developing follicle is associated with inhibition of follicular angiogenesis and antral follicular development, which results in the inhibition of ovulation (11, 12).

FSH is considered to be the fundamental driver of folliculogenesis. During the normal menstrual cycle, elevated FSH levels in the early follicular phase stimulate recruitment and growth of preantral and small antral follicles. In the mid- and late follicular phases, however, the decline of FSH concentrations and a progressive rise of LH levels are associated with the selection and growth of the dominant follicle destined for ovulation. Gonadotropins are even used in controlled ovarian stimulation (COS), which is an important component of assisted reproduction technology (ART). Particularly, exogenous FSH administration, alone or with variable amounts of LH activity, causes a rise of FSH concentrations throughout the follicular phase, so that the development of multiple ovarian follicles and oocytes is achieved (13). However, recent studies have shown that selective addition of LH activity, in the form of low-dose hCG, can replace mid- or late follicular phases' FSH administration (14).

CORPUS LUTEUM

Following ovulation, under the influence of luteogenic hormones, the corpus luteum (CL) develops from the remnants of the ovulated ovarian follicle. This process called luteinization and the stimulus for its initiation, the preovulatory LH surge, are common among species. The morphological events underlying this process involve intense reorganization of constituent cells, particularly granulosa cells, phenomena that includes varying cell-matrix interactions. These events, however, are poorly characterized. After expulsion of the oocyte, the blood capillaries of the theca rapidly invade the granulosa, thereby provoking the transformation of these cells (luteinization) and the formation of the CL. The blood vessels completely traverse the granulosa and open up in the follicular cavity. The granulosa cells are transformed into large luteal cells whose ultrastructure is the same as that of steroidogenic cells. The main hormone product of the CL is progesterone, which induces the necessary endometrial modifications required for the acquisition of a receptive state, an anticipation of embryo implantation. The life span of the CL is limited. In a nonfertile cycle, corpora lutea regress at the end of the menstrual cycle and are eliminated by a process called luteolysis. If pregnancy does occur, regression must be inhibited since the CL is the main source of steroidogenesis, supporting the establishment and maintenance of a successful pregnancy. Although some of the biochemical and endocrine events characterizing the formation and regression of CL have been well established, the molecular aspects underlying luteinized granulosa cell (GC) migration and survival and the endocrine/paracrine mechanisms by which LH and hCG act on GCs to transform the ruptured follicle into the CL are not well characterized. A number of studies have shown that cell-cell adhesion is strongly correlated with maturation and integrity of CL (15). It is also believed that VEGF and its receptor Flt-1, which is expressed on luteinizing GCs (Figure 1.1), are involved in CL development. Recent studies, in a rat model, have shown that suppression of VEGF resulted in nearly complete

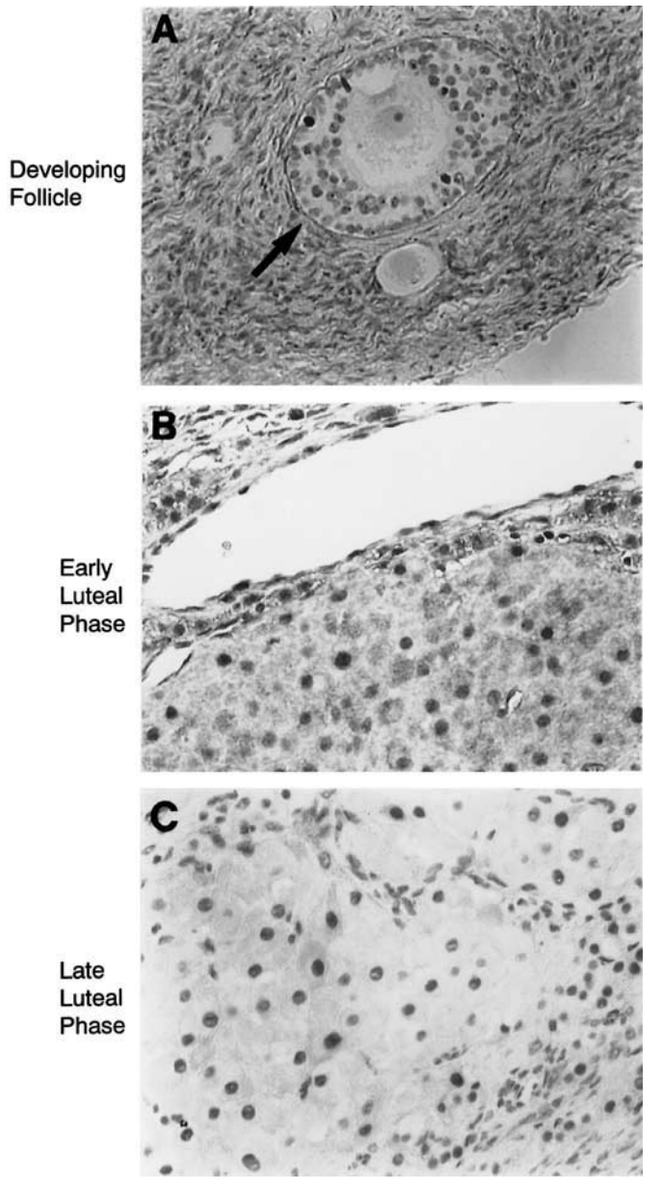

Figure 1.1. In situ staining of ovarian tissue for expression of Flt-1. Ovarian tissue was stained with antibodies against Flt-1 to determine its expression in developing follicles and during CL formation and regression. Flt-1 was not detected in developing follicles (A, arrows) but was present on the GCs of early luteal phase (B), expression that was not evident during the late luteal phase (C). (magnification: ×400).

suppression of CL formation (16). Our unpublished data extend these observations and support the idea that hCG promotes the migration and survival of human luteinized GCs, through a VEGF-dependent mechanism. Particularly, the following model is proposed (Figure 1.2): The binding of luteogenic hormone (LH or hCG) to GCs triggers their release of VEGF and induces the surface expression of VEGFR on these cells. The released VEGF (and possibly VEGF from other sources) in turn binds to the newly expressed VEGFR on the GCs, stimulating the secretion of FN into the surrounding matrix and upregulating the surface expression of at least two FN-binding

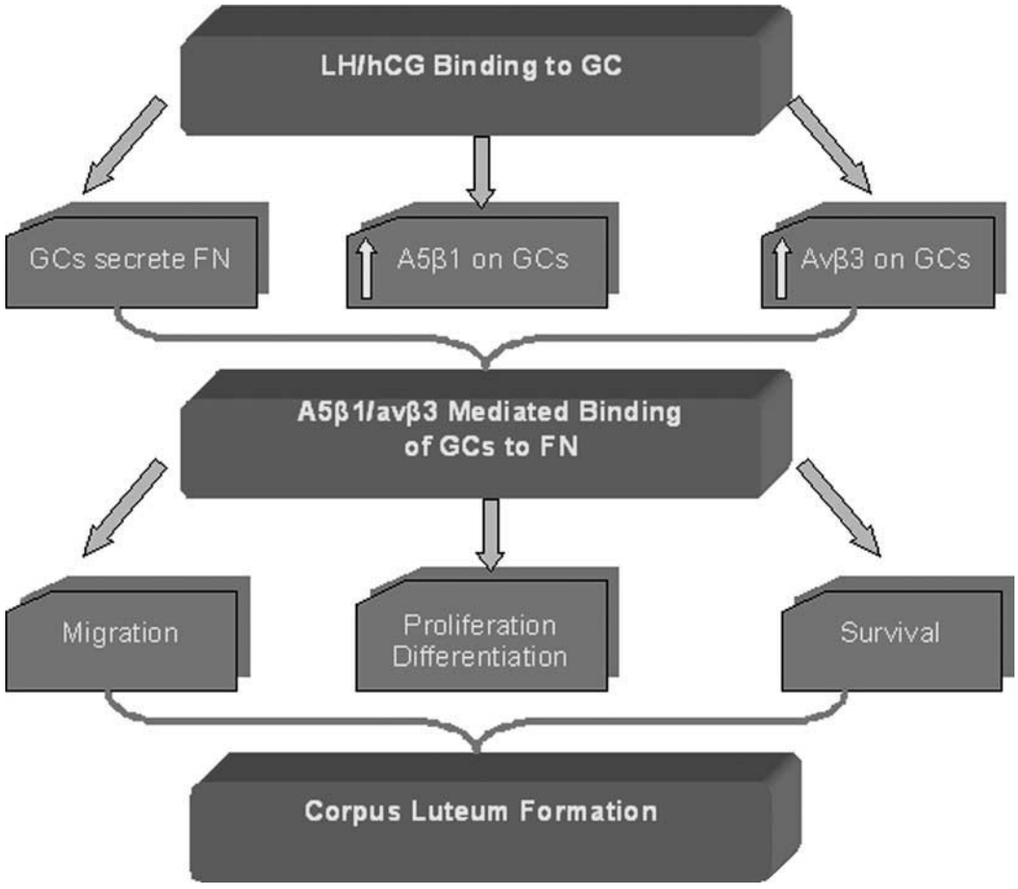

Figure 1.2. Proposed mechanism for the involvement of fibronectin and two of its integrin ligands in CL formation and their regulation by hCG through VEGF in this process.

integrins, $\alpha_5\beta_1$ and $\alpha_v\beta_3$. Subsequent interactions between FN and these integrins trigger adhesive events and intracellular signaling cascades involved in promoting the migration, survival, and differentiation of GCs, activities that contribute ultimately to the formation and/or persistence of the CL. Relative to atresia, little is known about luteolysis and the mechanisms that are involved in this process. Apoptosis seems to be the mechanism of CL regression in humans (17). While apoptosis is present already in the early CL, it is significantly increased in the late CL when luteal regression takes place (18). The Bcl-2 family members have been shown to play a central role in this process (19, 20).

Apoptosis and Apoptosis-Related Genes

It has been mentioned before that apoptosis or programmed cell death is an essential process in maintaining ovarian homeostasis in mammals and plays a prominent role in the development of fetal ovaries and in the postnatal ovarian cycle (21). It ensures that in every estrus/menstrus cycle, only one or very few follicles will ovulate. This process minimizes the possibility of multiple embryos during pregnancy. The rest of the follicles are gradually eliminated during the fertility period of the female. The apoptotic process of the old corpora lutea is essential for preserving the cyclicity and for ensuring the release of progesterone during the estrus/menstrus cycle (22). Furthermore, recent studies have shown that apoptosis of granulosa cells affects the conception in ovulation induction cycle (23) and that might explain the etiology of unexplained infertility (24). As is the case with other major organ systems, an evolutionarily conserved framework of genes and signaling pathways has been implicated in determining whether or not ovarian germ cells and somatic cells will die in response to either developmental cues or pathological insults. Therefore, it has been suggested that some apoptosis-related genes may have a role in ovarian follicular growth and atresia. The p53 gene is one of the most highly investigated tumor suppressor genes, and it seems to be a key player in apoptosis (25). The basic action of p53 is to protect the cellular genome from a variety of deleterious stimuli, such as reactive oxygen species and ionizing radiation. p53 is a transcriptional factor, which has the ability to alter the activity of target genes, an action that can be modulated by another antioncogenic protein, the product of the Wilms' tumour suppressor gene (*WT1*) (26). Regarding p53, nuclear accumulation of this tumor suppressor protein has been documented in GCs of follicles destined for atresia in the rat ovary, whereas in vivo gonadotropin priming inhibits granulosa cell apoptosis with a concomitant suppression of p53 immunoreactivity (27). These initial investigations have since been confirmed and extended by a number of laboratories, collectively supporting the hypothesis that nuclear translocation of p53 in GCs heralds their demise during follicular atresia (28).

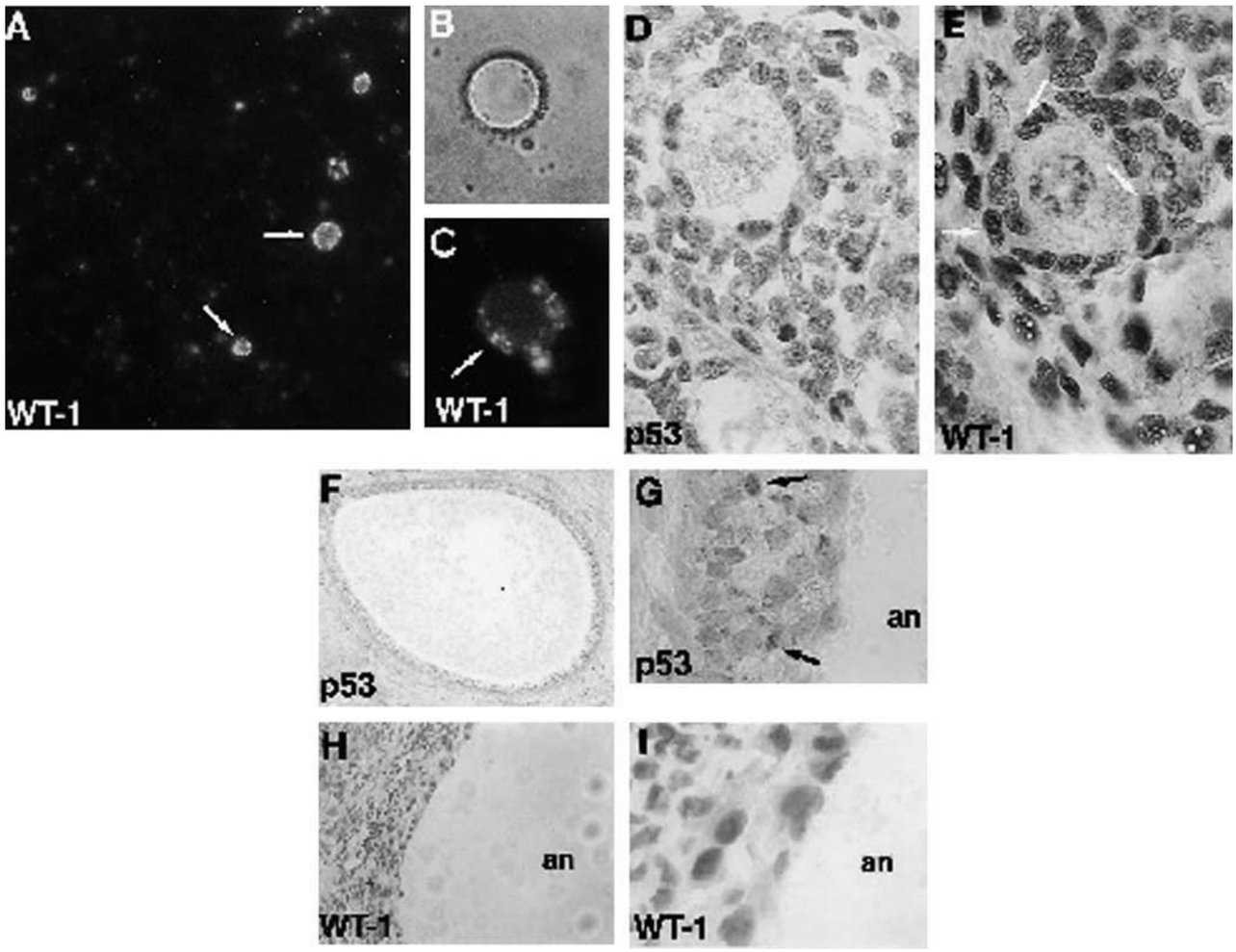

Figure 1.3. Expression of p53 and WT1 in the human ovary in situ. (A, B, and C) Immunolocalization of WT1 in isolated preantral human follicles. Human preantral follicles were isolated from ovarian biopsy specimens and immunostained for WT1. Under low magnification (A), multiple preantral follicles of varying sizes *(arrows*) can be seen surrounded by WT1-positive GCs. Under high magnification (B, phase contrast), remaining adherent GCs stain intensely for WT1 (C, *arrow*). Immunohistochemical detection of p53 (D) and WT1 (E) in fetal human ovary in situ. Note that p53 staining is absent (D) in contrast to the very strong staining of WT1 in almost all GCs (E, *arrows*). Immunohistochemical detection of p53 in an atretic follicle (F) and at a higher magnification (G); WT1 staining (H) and at a higher magnification (I) in an atretic follicle. Note that in the representative atretic follicle, p53 nuclear staining and some cytoplasmic staining were present in many GCs (G, *arrow*) in contrast to the absence of WT1 (H and I) in an antrum. Magnification: A and F, ×3100; B and C, ×3600; D, E, G, and I, ×3400; H, ×3200.

That p53 serves a similar function in the human ovary is suggested by the findings of p53 expression in the human ovary and isolated granulosa cells (29), as well as by recent studies on the ability of overexpressed p53 to induce apoptosis in transformed human granulosa cell lines (30). However, the spatial localization of p53 in the human ovary during follicular development and the regulation of tumor suppressor gene expression in nontransformed human GCs remain to be determined. Evidence linking these two important members of the tumor suppressor gene family, p53 and WT1, to ovarian follicular growth and atresia has been provided (31). It was shown that the p53 gene is expressed in GCs and is closely related to their survival. This apoptosis-inducing gene is reduced by treatment with exogenous gonadotropin in vivo (27), suggesting that p53 may play an important role in regulating follicular survival during gonadotropin-dependent stages of follicular life or maintenance of luteal cells during pregnancy. WT1, a known transcriptional regulator of p53, is also expressed in human GCs (31). Data that we have published indicate that this gene is constitutively expressed in human granulosa cells and its expression remains strong during early stages of development but progressively declines during gonadotrophin-dependent follicular maturation. These data suggest a possible implication of WT1 in the mechanisms responsible for the maintenance of a quiescent state in follicles during gonadotropin-independent stages of follicular life (31) (Figure 1.3). Many p53-regulated target genes have been identified, including the *bcl-2* prosurvival gene, the *bax* proapoptotic gene, and several reduction-oxidation genes. In particular, it is believed that interaction among pro- and antiapoptotic members of the bcl-2 family in the mitochondrion determines whether or not the apoptotic pathways are activated. This interaction regulates release of

cytochrome *c* from the mitochondrion into the cytosol, which activates caspase-9 and downstream caspases, including caspase-3 (32). Bcl-2 gene was first discovered by its involvement in B-cell malignancies. A growing number of studies have implicated this proto-oncogene in apoptotic events of ovarian follicles in the different developmental stages. In the recent years, it has been demonstrated that Bcl-2 is an important factor in regulating apoptosis of human GCs (33), but the role of Bcl-2 in ovarian function remains to be fully elucidated. Bax gene is another member of the bcl-2 gene family, which can induce apoptosis through inactivation of bcl-2 in cells. Targeted disruption of the bax gene in mice leads to a defect in the ability of GCs to undergo apoptosis during follicular atresia (34), while a reduction in bax levels was correlated with gonadotropin-mediated follicular survival in rat GCs (27). Bax has also been detected in human apoptotic GCs, but not in healthy follicles (29), even though its precise role in the development and apoptosis of human follicles is not yet understood.

Role of Steroids

Sex steroids play an important role in the development of the ovarian follicle and in the preservation of fertility in women (35). In the ovary, steroids are produced by theca and granulosa cells, and this process is induced by gonadotropins. The well-known ''two-cell, two-gonadotrophin'' theory emphasizes the fact that stimulation of both theca cells by LH and granulosa cells by FSH is required for estradiol synthesis. Progesterone (P4) is one of the major steroids secreted by the ovary, and it is synthesized by preantral follicles. The rate of secretion is increased while follicular development proceeds, and many studies have shown the importance of these high P4 levels in regulating ovulation (36). It has been suggested that progesterone, and other ovarian steroids, may act upon the ovary in part through influences on cGMP concentrations (37). Progesterone production is induced by LH as it has been shown in rat and porcine cultured GCs, whereas progesterone antagonist, RU486, inhibits luteinization of GCs coming from preovulatory follicles (38). Moreover, it has recently been indicated that progesterone is involved in stimulating ovulation in human ovarian follicles (39), probably by inducing production of proteolytic enzymes, important for ovulating process. Progesterone also acts through the progesterone receptor to inhibit GC/luteal cell apoptosis (40). We have also shown that RU486 triggers apoptosis in human GC. Androgens are produced by theca cells in response to LH and exert their actions via receptors localized to secondary and dominant follicles in human. Prenatal testosterone treatment in ewes indicates an enhancement in follicular development (41), whereas treatment with androgens in primate ovaries led to an increased number of small antral and preantral follicles (42). Moreover, androgens are thought to play a role in oocyte maturation by enhancing granulosa cells' differentiation (43). In PCOS patients, abnormal androgen production, by theca cells, leads to hyperandrogenism, which is thought to be responsible for anovulation (44). This hypothesis is supported by reports from therapies with the antiandrogens, flutamide, or cyproterone acetate, where ovulation was restored and the rates of pregnancy were extremely higher (45). However, the precise mechanisms that lead to anovulation in a large number of PCOS patients are very complex and need to be elucidated in the future. Estrogens are also produced in the ovary by aromatization of androgens. In particular, androgens are the substrate of P450 aromatase, an enzyme that mediates the conversion to estrogens. Estradiol exerts its actions via two forms of receptors, ERa and ERb. Both isoforms are important for the female reproductive ability, but ERb receptor has shown to be implicated in follicular growth (46). In normal ovaries, increased estrogen production by the dominant follicles leads to a decrease in FSH serum concentration due to negative feedback effects of estradiol on the hypothalamipituitary axis. The reduction in FSH levels inhibits the development of less mature follicles, but whether estrogens are essential for follicular growth and oocyte maturation remains unclear yet.

Role of Adhesion Molecules

Follicular development is established by gap junctions and adhesion-type junctions between GCs. The degeneration of the follicles and the CL regression are associated with the loss of adherence between GC. Previous studies have also revealed that a single large GC is twice as likely to be apoptotic after culture, as an aggregated GC (47). This observation implies that cell contact may inhibit GC apoptosis. Despite the fact that aggregated GCs are more steroidogenic, there is evidence that progesterone production is not associated with the mechanism by which cell contact prevents apoptosis of GCs. It was also been found that gap junctions are not involved with the survival of GCs, and this observation supported the idea that the adhesion-type junctions convey the protective effects of cell contact. Cell-to-cell contact is mediated by a great diversity of cell adhesion molecules including some integrins, the immunoglobulin supergene family, selectins, and cadherins. The expression of these adhesion molecules is cell specific, with cadherins involved in mediating calcium-dependent cell-to-cell adhesion in virtually all solid tissues of multicellular organisms (48). Cadherins are a rapidly expanding family of calcium-dependent CAMs and have been shown to regulate epithelial, endothelial, neural, and cancer cell adhesion, with different CADs expressed on different cell types. The adhesion junctions are formed between adjacent cells through a homophilic interaction of N-cadherin molecules, which is expressed in primordial, primary, and early secondary follicles, as well as in healthy antral follicles (47) and is located at the junctional interface between aggregating cells. Luteal cells are also strongly positive for N-cadherin in the early luteal and midluteal phase, whereas there is only weak N-cadherin staining during late luteal phase. As the follicle degenerates, the expression of N-cadherin decreases, and GCs ultimately dissociate (47). In addition, apoptosis does not occur in preantral follicles and is very low in the early luteal phase, whereas it increases significantly in the late luteal phase (47). These observations suggest (a) that the expression of N-cadherin is regulated in human GCs in vivo during follicular maturation and CL formation and (b) that there is a direct correlation between the presence of the N-cadherin molecule and the absence of features characteristic of cellular apoptosis. The mechanism through which N-cadherin exerts the survival effects on GCs is not yet completely understood. Recent studies have shown that N-cadherin interacts with the FGF receptor, which is required for cell contact to prevent apoptosis (49). Additionally, bFGF induces the tyrosine phosphorylation of its own receptor by

inducing PKC activity, a process that is involved in stimulating calcium uptake into the cytoplasmic stores (50). Taking these data together, it is likely that N-cadherin interaction with FGF receptor promotes cell survival by enhancing the activity of PKC and thereby maintaining calcium homeostasis (51). Moreover, it have been demonstrated that cell-cell aggregation promotes survival of GCs and that loss of N-cadherin from the cell surface induces apoptosis in these cells, supporting a major role of this adhesion molecule in the GC life cycle. N-cadherin possesses an extracellular domain with five tandemly arranged repeats. The N-terminal repeat contains the adhesive domain that is involved in cadherin-specific adhesions. Cleavage of the extracellular domain by metalloproteinases (MMP) is followed by loss of the adhesive ability of N-cadherin and cell death. It has been shown that inhibition of cleavage by MMP inhibitor decreases the rate of apoptosis in granulosa cells (47). Other studies have indicated cAMP-dependent pathways that induce downregulation of N-cadherin in a dose-dependent manner.

E-cadherin is also an important member of the cadherin superfamily, and it is expressed in spontaneously immortalized granulosa cells (SIGC) (52). E-cadherin connects adjacent cells, and a disruption in calcium-dependent cell contacts, with EGTA or an E-cadherin antibody, results in an increase in caspase-3 activity, in both the cytoplasm and nuclei of SIGCs. There have been detected cleavage products of β-catenin, which is an E-cadherin-associated protein, in apoptotic SIGCs. Previous studies have revealed that β-catenin and E-cadherin are substrates for caspase-3. These findings strengthened the idea that the increase of the cytoplasmic caspase-3 activity is associated with the degradation of β-catenin and E-cadherin. It is thus presumed that promotion of cell survival by E-cadherin is regulated by a signal transduction pathway that inhibits the activation of caspase-3.

CONCLUSIONS

Follicular development and CL formation and regression in human ovaries are strongly correlated with female reproductive capacity. Production of steroids and apoptosis of ovarian cells seems to play an active and important role in ovarian physiological functions. Disruption of the normal activity of the ovary may lead to reproductive disorders or even malignancies. Therefore, the mechanisms that control the normal life cycle of the dominant follicle, from folliculogenesis to luteolysis, must be elucidated.

KEY POINTS

- Development of multiple ovarian follicles and oocytes is achieved in controlled ovarian stimulation, which is an important component of assisted reproduction technology.
- The main hormone product of the CL is progesterone, which induces the endometrial modifications required for embryo implantation.
- Progesterone is also involved in stimulating ovulation in human ovarian follicles.
- In PCOS patients, abnormal androgen production, by theca cells, leads to hyperandrogenism, which is thought to be responsible for anovulation.
- Estrogen production by the dominant follicle leads to a decrease in FSH levels, which inhibits the development of less mature follicles.
- Apoptosis or programmed cell death ensures that in every estrus/menstrus cycle, only one or very few follicles will ovulate. This process minimizes the possibility of multiple embryos during pregnancy.

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2

MECHANISMS OF FOLLICULAR DEVELOPMENT: THE ROLE OF GONADOTROPHINS

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INTRODUCTION

Folliculogenesis in women is a dynamic and uninterrupted process from fetal life until menopause. Following pubertal maturation of the reproductive axis, all types of follicles from the primordial to the preovulatory stage are present in the human ovary. Over the past twenty years, it has become clear that these follicles represent sequential forms of the developmental process classified into eight categories, based on the size and the number of the granulosa cells (Gougeon, 1986). For example, class 1 corresponds to a secondary preantral follicle and class 8 to a large preovulatory follicle.

Folliculogenesis is a lengthy process (Figure 2.1). Based on the calculation of the doubling time of granulosa cells, it is estimated that the time spent from the primordial to the preovulatory stage is approximately one year (Gougeon, 1986). However, maturation of a follicle from class 1 to class 8 is achieved within eighty-five days (Gougeon, 1986). At the beginning, proliferation of the granulosa cells on several layers takes place and the primordial follicle becomes preantral. Following this, the theca interna develops and the antral cavity is formed. The rate at which follicles leave the primordial pool is not known. However, it seems that the departure follows an ordered sequence, so that follicles formed first leave the pool earlier (Hirshfield, 1991).

It remains unclear which factors are responsible for the initiation of maturation of a primordial follicle or what is the trigger for the passage of a follicle from the preantral to the antral stage (Figure 2.1). In humans, this part of folliculogenesis is gonadotrophin independent. The growth of a follicle from class 1 to class 5 is to some extent affected by gonadotrophins, while from class 5 to class 8, that is, during the last fifteen days of follicle maturation that correspond to the follicular phase of the normal menstrual cycle, gonadotrophins are the only determinants of follicle growth (Gougeon, 1986). In other words, follicle maturation to the preovulatory stage is not feasible without the presence of follicle-stimulating harmone (FSH) and luteinizing harmone (LH).

INITIAL RECRUITMENT: PREANTRAL FOLLICLE GROWTH

The term ''recruitment'' refers to a cohort of follicles that leave a particular developmental stage for further growth (Figure 2.1). At the primordial stage, the term ''initial recruitment'' has been proposed (McGee and Hsueh, 2000). Similarly, the term ''cyclic recruitment'' has been proposed for the cohort of antral follicles from which ''selection'' of the dominant follicle takes place during the early follicular phase of the cycle (McGee and Hsueh, 2000). It has been established that FSH is the principal hormone that promotes follicle maturation, especially at more advanced stages of development. Although receptors of FSH are expressed in the granulosa cells of preantral follicles (Roy et al., 1987), evidence has been provided that in humans this hormone is not required for follicle maturation up to the antral stage. A logical explanation is that primordial follicles are located in an avascular part of the ovary, and therefore, they can be easily reached by locally produced but not by systemic factors (van Wezel and Rodgers, 1996). There are several examples of follicle maturation up to the early antral stage in women either in the presence of negligible amounts of FSH in the circulation, such as before puberty (Peters et al., 1978), during pregnancy (Westergaard et al., 1985), and in cases of hypogonadotrophic hypogonadism (Rabin et al., 1972), or in the absence of FSH activity, such as in mutations of FSHβ (Matthews et al., 1993) and inactivating mutations of the FSH receptor (Touraine et al., 1999).

The situation is different in certain species in which FSH participates in the control of preantral follicle development, although it is still unclear whether this hormone is involved in the mechanism that triggers initial recruitment. In vitro data have demonstrated that FSH is a growth and differentiation factor for rat preantral follicles in the presence of a cGMP analog that suppresses apoptosis (McGee et al., 1997). However, FSH alone did not prevent apoptosis in these follicles. Also, preantral follicle growth, number of cells, and cell differentiation were promoted by FSH, and these effects were enhanced by the addition of antimullerian hormone (AMH or MIS) or activin (McGee et al., 2001). In contrast to rats, in mice, AMH, produced by preantral follicles, inhibited initial recruitment as well as the stimulatory effect of FSH on the growth from the primary to the early antral stage (Visser and Themmen, 2005). Although still unclear, species variability may account for these opposite actions of AMH. Recently, AMH was able to inhibit initiation of human primordial follicle growth in vitro (Carlsson et al., 2006).

Several genes encoding specific proteins and growth factors are expressed in the granulosa cells of small follicles. These factors including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), TGF-β and insulin-like growth factor-1 (IGF-1) may be involved in the initiation of growth

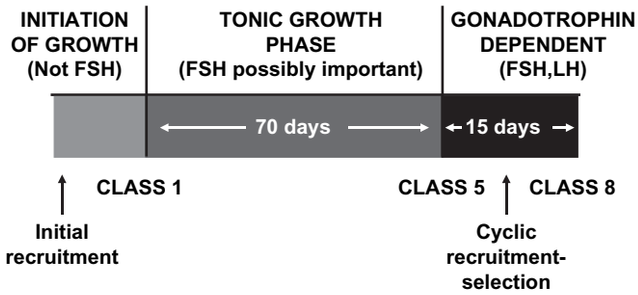

Figure 2.1. Growth of follicles through different stages and duration of follicle maturation. Class 1 corresponds to the secondary preantral follicle and class 8 to the large preovulatory follicle. The period from class 5 to class 8 corresponds to the follicular phase of the normal menstrual cycle and is gonadotrophin dependant. "Initial recruitment" refers to recruitment of primordial follicles, while "cyclic recruitment" refers to the recruitment and selection of the dominant follicle. The diagram is based on data presented in one reference (Gougeon, 1986).

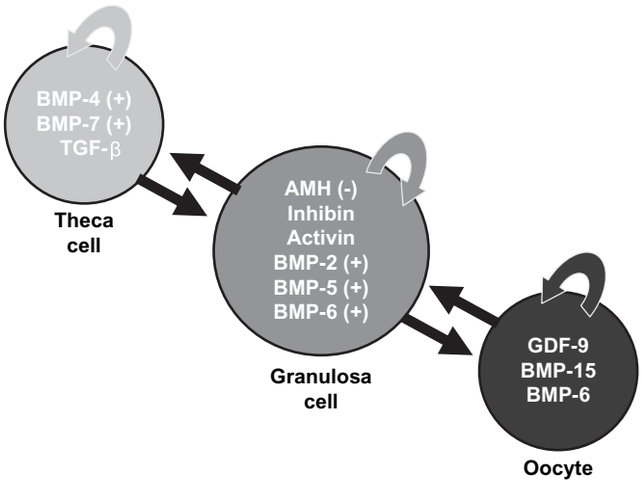

Figure 2.2. Members of the superfamily of TGF-β predominantly produced by the theca cells, granulosa cells, and the oocyte. Paracrine effects are exerted between the theca and the granulosa cells as well as between the granulosa cells and the oocyte. Autocrine effects are also likely. The diagram is based on data presented in one reference (Knight and Glister, 2006).

of primordial follicles (May et al., 1990; Adashi, 1998; Knight and Glister, 2006). A group of such factors, members of the TGF-β superfamily, are shown in Figure 2.2. Another factor, produced particularly in primordial and primary follicles, is Wilms tumor suppressor gene (*WT1*) that may have a suppressive role in the expression of genes of various growth factors, maintaining thus the follicles at the resting stage (Hsu et al., 1995). Similarly, retinoblastoma protein (pRb) may also have a suppressive effect by inhibiting proliferation of granulosa cells even in humans (Bukovsky et al., 1995a, 1995b). A role in the transition from the primordial to the primary stage may be also played by the oncogene *myc* (Piontkewitz et al., 1997), the *steel* locus encoding the kit ligand (Huang et al., 1993), and the platelet-derived growth factor (Nilsson et al., 2006). Finally, neurotrophin molecules, such as nerve growth factor (NGF) may also play a role in mice for early follicular development (Dissen et al., 2001).

During follicle growth, the oocyte undergoes functional changes in order to become fertilizable at ovulation. However, various factors derived from the oocyte seem to play a crucial role for preantral follicle development (Erickson and Shimasaki, 2001). For example, growth differentiation factor-9 (GDF-9), a member of TGF-β superfamily, is expressed by oocytes (McGrath et al., 1995) and stimulates granulosa cell proliferation and DNA synthesis in preantral and dominant follicles (Vitt et al., 2000). This factor also stimulates preantral follicle growth in vitro (Hayashi et al., 1999). It has been hypothesized that a concentration gradient of GDF-9 is established in the graafian follicle with the highest levels nearest the oocyte (Erickson and Shimasaki, 2000). This gradient in GDF-9 concentrations influences granulosa cell differentiation in response to FSH stimulation that leads to granulosa cell heterogeneity. For example, in the membrana cells, the expression of genes encoding LH receptors, P450 aromatase, P450 side chain cleavage, kit ligand, and urokinase-type plasminogen activator is facilitated by GDF-9, while in the cumulus cells, genes encoding IGF-1, hyaluronic acid synthase-2, and cycloxygenase-2 (COX-2) are expressed (Erickson and Shimasaki, 2000). Nevertheless, the effects of GDF-9 are different in preantral and dominant follicles.

Another protein that is also produced by the oocyte is bone morphogenetic protein-15 (BMP-15 or GDF-9B) (Dube et al., 1998). Similar to GDF-9, this protein stimulates granulosa cell mitosis in vitro (Otsuka et al., 2000). Furthermore, BMP-15 promotes early follicle growth but restrains the transition of follicles to the preovulatory stage, while it inhibits the expression of FSH receptors and prevents premature luteinization (Moore and Shimasaki, 2005). GDF-9 and BMP-15 are obligatory for normal folliculogenesis and female fertility. GDF-9 knockout mice remain infertile, with follicles being arrested at the primary stage (Carabatsos et al., 1998). Similarly, in sheep homozygous for point mutations of BMP-15 or GDF-9 genes or in animals immunized against these two factors, follicles fail to develop beyond the primary stage (McNatty et al., 2005). Finally, in women, a BMP-15 mutation causes ovarian dysgenesis with hypergonadotrophic ovarian failure (Di Pasquale et al., 2004). Apart from these two factors, other substances produced by the oocyte, such as BMP-6, fibroblast growth factor-8 (FGF-8), and TGF-β2, have been investigated to a lesser extent (Erickson and Shimasaki, 2000). Nevertheless, BMP-4 and BMP-7 produced by ovarian stroma and theca cells have been associated with the transition of follicles from the primordial to the primary stage (Knight and Glister, 2006).

GROWTH OF ANTRAL FOLLICLES: DOMINANT FOLLICLE

FSH Intercycle Rise

The growth of large antral follicles is depended on gonadotrophins. These hormones are obligatory for follicle maturation during the follicular phase of the cycle. Despite the apparently low FSH levels in the follicular and the luteal phase of the cycle (Messinis and Templeton, 1988a), important changes in the secretion of this hormone take place during the luteal-follicular transition. In particular, FSH levels increase in late luteal phase up to the onset of menstruation and the early follicular phase of the following cycle (Mais et al., 1987; Messinis et al., 1993).