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Initial and Cyclic Recruitment of Ovarian Follicles*

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ABSTRACT

Mammalian ovaries consist of follicles as basic functional units. The total number of ovarian follicles is determined early in life, and the depletion of this pool leads to reproductive senescence. Each follicle develops to either ovulate or, more likely, to undergo degeneration. The dynamics of ovarian follicle development have interested endocrinologists and developmental biologists for many years. With the advent of assisted reproductive techniques in humans, the possibility of regulating follicle development *in vivo* and *in vitro* has gained clinical relevance. In this review, we focus upon key branching points during the development of ovarian follicles as well as factors involved in determining the eventual destiny of individual follicles. We discuss inconsistencies in the literature regarding the definitions of follicle recruitment and selection and propose to name the two major steps of follicle development as initial and cyclic recruitment, respectively. Because some of these disparities have arisen due to differences in the animal systems studied, we also compare the development of the ovarian follicles of both humans and rats. We also review the status of knowledge of several puzzling clinical issues that may provide important clues toward unlocking the mechanisms of follicle development. (*Endocrine Reviews* **21:** 200–214, 2000)

I. Life History of Ovarian Follicles

A. Follicle endowment, growth, demise, and ovulation

THE MAJOR function of the female gonad is the differentiation and release of the mature oocyte for fertilization and successful propagation of the species. Additionally, the ovary produces steroids that allow the development of female secondary sexual characteristics and support pregnancy. In mammalian ovaries the individual follicles consist of an innermost oocyte, surrounding granulosa cells, and outer layers of thecal cells. The fate of each follicle is controlled by endocrine as well as paracrine factors (1–5). The follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas a few of them, under the cyclic gonadotropin stimulation that occurs after puberty, reach the preovulatory stage (1, 2). These Graafian follicles are the major source of the cyclic secretion of ovarian estrogens in women of reproductive age. In response to preovulatory gonadotropin surges during each reproductive cycle, the dominant Graafian follicle ovulates to release the mature oocyte for fertilization, whereas the remaining theca and granulosa cells undergo transformation to become the corpus luteum (6). The pool of oocytes in the mammalian ovary becomes fixed early in life; thus, ovarian senescence is linked to the dwindling supply and eventual exhaustion of the pool of primordial follicles (Fig. 1).

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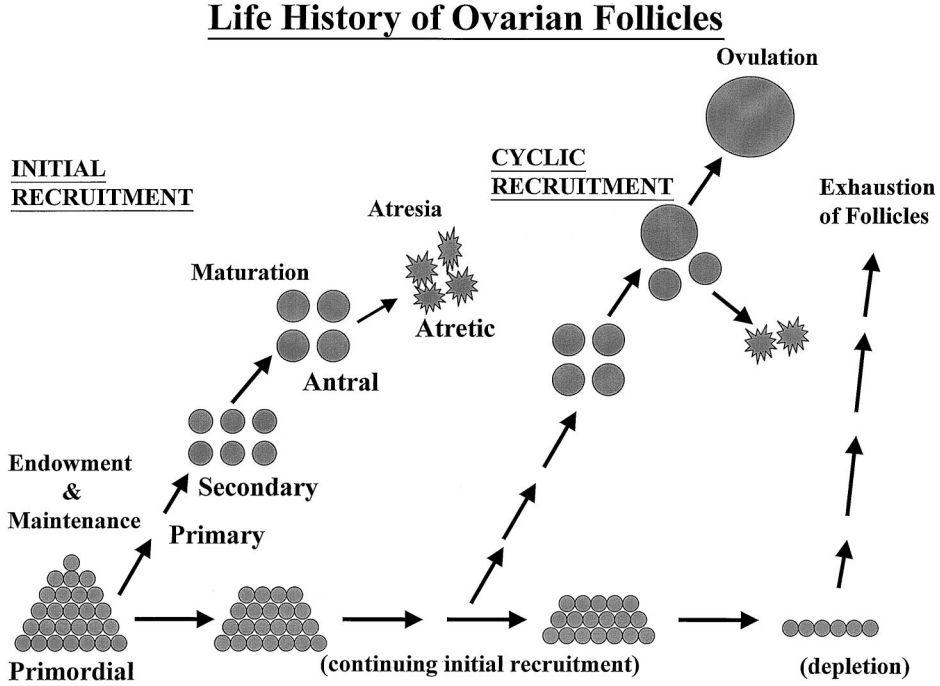

FIG. 1. Life history of ovarian follicles: endowment and maintenance, initial recruitment, maturation, atresia or cyclic recruitment, ovulation, and exhaustion. A fixed number of primordial follicles are endowed during early life, and most of them are maintained in a resting state. Growth of some of these dormant follicles is initiated before and throughout reproductive life (Initial recruitment). Follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage most follicles undergo atresia; however, under optimal gonadotropin stimulation that occurs after puberty, a few of them are rescued (Cyclic recruitment) to reach the preovulatory stage. Eventually, depletion of the pool of resting follicles leads to ovarian follicle exhaustion and senescence.

TABLE 1. Differences between initial and cyclic recruitment of ovarian follicles

	Initial recruitment (initiation of growth)	Cyclic recruitment (escape from atresia)
Stages	Primordial	Antral (human: 2–5 mm in diameter; rodents: 0.2–0.4 mm in diameter)
Hormones involved	Not determined	FSH
Default pathway	Remain dormant	Apoptosis
Timing	Continuous throughout life, begins after follicle formation	Cyclic (human: 28 days, rodents: 4–5 days), starts after puberty onset
Oocyte status	Starting to grow, not capable of undergoing germinal vesicle breakdown	Completed growth, competent to undergo germinal vesicle breakdown

B. Initial vs. cyclic recruitment

The term recruitment has been used frequently by different investigators to describe two important but distinct decision points during follicle development (7–10). The dormant primordial follicles are *recruited* into the growing follicle pool in a continuous manner, whereas increases in circulating FSH during each reproductive cycle *recruit* a cohort of antral follicles. To avoid confusion, we propose designating these branching points as *initial* recruitment and *cyclic* recruitment and have summarized major differences between the two processes in Table 1.

During initial recruitment, intraovarian and/or other unknown factors stimulate some primordial follicles to initiate growth, whereas the rest of the follicles remain quiescent for months or years. Alternately, initial recruitment may be due to a release from inhibitory stimuli that maintain the resting follicles in stasis. Initial recruitment is believed to be a continuous process that starts just after follicle formation, long before pubertal onset. After initial recruitment, oocyte growth is a prominent feature of the growing follicles, but these oocytes remain arrested in the prophase of meiosis. For those follicles not recruited, the default pathway is to remain dormant.

In contrast, cyclic recruitment starts after pubertal onset and is the result of the increase in circulating FSH during each reproductive cycle that rescues a cohort of antral follicles from atresia. In rodents, the recruitable early antral follicles are 0.2–0.4 mm in diameter, whereas human follicles at the comparable stage are larger (2–5 mm in diameter) and have acquired antrum for some time. During cyclic recruit-

ment, only a limited number of follicles survive, and the default pathway is to undergo atresia. Oocytes in these follicles have already completed their growth, acquired a zona pellucida, and are competent to resume meiosis (11, 12).

C. Negative and positive selection leading to dominance

Similar to the confusion associated with two distinct stages of follicle recruitment, the FSH-initiated cyclic recruitment step is sometimes described interchangeably with the process of follicle selection (9, 10, 13). Cyclic recruitment and selection of follicles represent a continuous process, eventually leading to the emergence of the preovulatory follicle(s). Cyclic recruitment and final follicle selection are most clearly illustrated during the human menstrual cycle (Fig. 2). After increases in circulating FSH during the perimenstrual period, a cohort of antral follicles escapes apoptosis due to the survival action of FSH. Among this group of about 10 antral follicles (found in young adults), one of the leading follicles grows faster than the rest of the cohort and produces higher levels of estrogens and inhibins (4). Although the exact reasons why one follicle emerges as dominant are unclear, this follicle is likely to be more sensitive to FSH (6), perhaps because of enhanced FSH and/or LH receptor expression or increases in local growth factors that augment FSH responsiveness as suggested by bovine studies (14–16). Estrogens and inhibins produced by the largest follicle suppress pituitary FSH released during the midfollicular phase. As a result, the remaining growing antral follicles are deprived of adequate FSH stimulation required for survival (17). In monkeys, it has been elegantly demonstrated that immunoneutralization of the actions of circulating estrogens during the midfollicular phase leads to sustained elevation of circulating FSH, thus allowing the development of multiple preovulatory follicles (18). Furthermore, administration of exogenous estrogens suppresses follicle development in women (19, 20), whereas treatment with high levels of exogenous gonadotropins during ovulation induction in women is widely known to stimulate the growth of multiple preovulatory follicles (6).

Negative selection against subordinate follicles is therefore a result of estrogen and inhibin produced by the dominant follicle exerting negative feedback upon gonadotropin release. Additionally, this rapidly growing follicle also produces higher levels of autocrine and paracrine growth factors that stimulate increases in vasculature and FSH responsiveness, thus constituting a local positive selection mechanism. Multiple studies have demonstrated the importance of insulin-like growth factors (IGFs) and other local factors in the amplification of FSH action (21, 22). Although remaining to be characterized, atretogenic factors produced by the dominant follicle have been postulated to account for the lack of development of subordinate follicles after exogenous gonad-

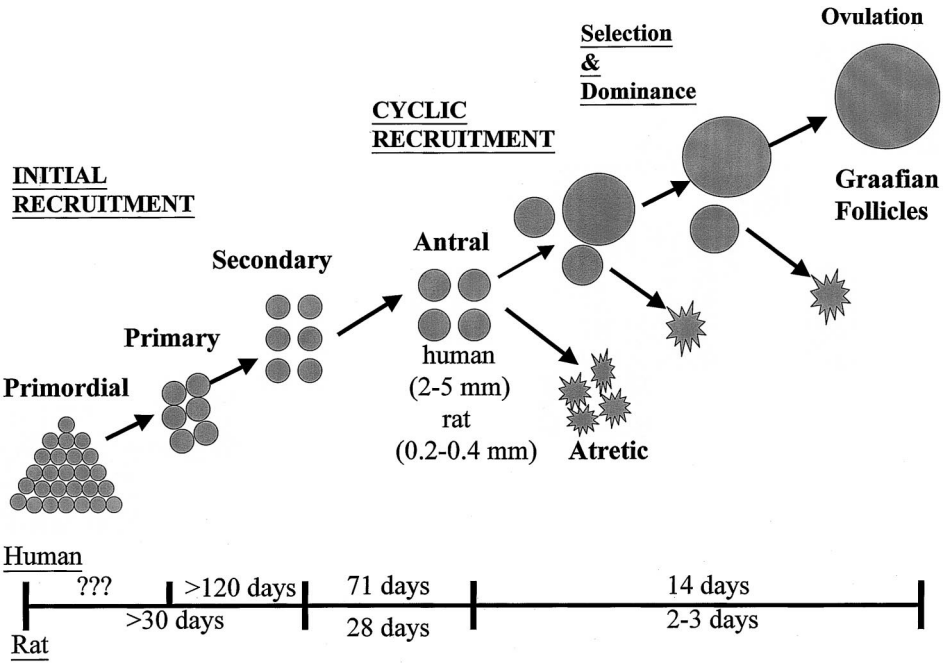

FIG. 2. Duration of follicle recruitment and selection in human and rat ovaries. Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles. Due to its protracted nature, the duration required for this step is unknown. In the human ovary, greater than 120 days are required for the primary follicles to reach the secondary follicle stage, whereas 71 days are needed to grow from the secondary to the early antral stage. During cyclic recruitment, increases in circulating FSH allow a cohort of antral follicles (2–5 mm in diameter) to escape apoptotic demise. Among this cohort, a leading follicle emerges as dominant by secreting high levels of estrogens and inhibins to suppress pituitary FSH release. The result is a negative selection of the remaining cohort, leading to its ultimate demise. Concomitantly, increases in local growth factors and vasculature allow a positive selection of the dominant follicle, thus ensuring its final growth and eventual ovulation. After cyclic recruitment, it takes only 2 weeks for an antral follicle to become a dominant Graafian follicle. In the rat, the duration of follicle development is much shorter than that needed for human follicles. The time required between the initial recruitment of a primordial follicle and its growth to the secondary stage is more than 30 days, whereas the time for a secondary follicle to reach the early antral stage is about 28 days. Once reaching the early antral stage (0.2–0.4 in diameter), the follicles are subjected to cyclic recruitment, and only 2–3 days are needed for them to grow into preovulatory follicles.

otropin administration (9, 23). Furthermore, the increased responsiveness of dominant follicles to FSH stimulates the expression of both FSH and LH receptors in the granulosa cells of this follicle (24, 25), thus providing a fail-safe mechanism to ensure the eventual ovulation of the selected follicle. Recent computer modeling of ultrasound images in patients also suggested a suppressive effect of the dominant follicle on its neighboring subordinate ones (26).

Cyclic recruitment of early antral follicles and selection of dominant follicles in rodents is similar to that of the primates with the major exception that multiple follicles become dominant during each estrous cycle. Monoovulatory and polyovulatory species likely differ in the threshold (or set point) for negative feedback signals, presumably a genetically determined trait (27, 28). The law of follicular constancy proposed by Lipschutz (29) emphasizes that the ovulatory number remains constant in a given species even when a single ovary or a large portion of the remaining ovary is removed. Thus, findings of compensatory ovulation (30) underline the importance of the putative central set point within a given species. In several high fecundity strains of sheep, the follicular negative feedback signals (estrogens and inhibins) secreted by each individual follicle are decreased, thus allowing the selection of more preovulatory follicles (31). In general, the preovulatory dominant follicles in these animals are of a smaller size than those found in the low fecundity strain (32–34). Furthermore, species and strain differences in follicular responsiveness to FSH (35), or the available number of growing antral follicles, may also play a role in determining the number of preovulatory-size follicles.

D. Chronicle of early follicle development in humans

In humans, primordial germ cells arrive in the gonadal ridge from the yolk sac endoderm by the seventh week of gestation to become oogonia, which proliferate by mitosis before differentiating into primary oocytes. Some oogonia begin transformation into primary oocytes and enter the first stages of meiosis at around 11–12 weeks of gestation (Fig. 3). The total germ cell number peaks at 20 weeks. After this time, the rate of oogonial division declines. Primordial follicle formation begins around midgestation when a single layer of pregranulosa cells surround each oocyte and continues until just after birth (36). After oocytes are within the primordial follicles, they remain arrested in the dictyate stage of meiosis I. From a peak of 6 to 7 million at 20 weeks of gestation, the oocyte number falls dramatically so that at birth, there are only 300,000 to 400,000 remaining (37, 38). Oocytes not surrounded by granulosa cells to form primordial follicles are lost, probably via apoptosis (39–41). Meanwhile, some primordial follicles leave the resting pool by initiating growth (Fig. 2; initial recruitment). Once entering the growing pool, most growing follicles progress to the antral stage, at which point they inevitably undergo atresia. After pubertal onset, a small number of the antral follicles can be rescued by gonadotropins to continue growth (Fig. 2; cyclic recruitment), and normally one Graafian follicle is formed each month in preparation for ovulation. Antral follicles (2–5 mm in diameter) develop into Graafian follicles in only 14 days during the follicular phase of the menstrual cycle, although more than 85 days are needed for late secondary follicles to grow into preovulatory follicles (1) (Fig. 2). In addition, it has been esti-

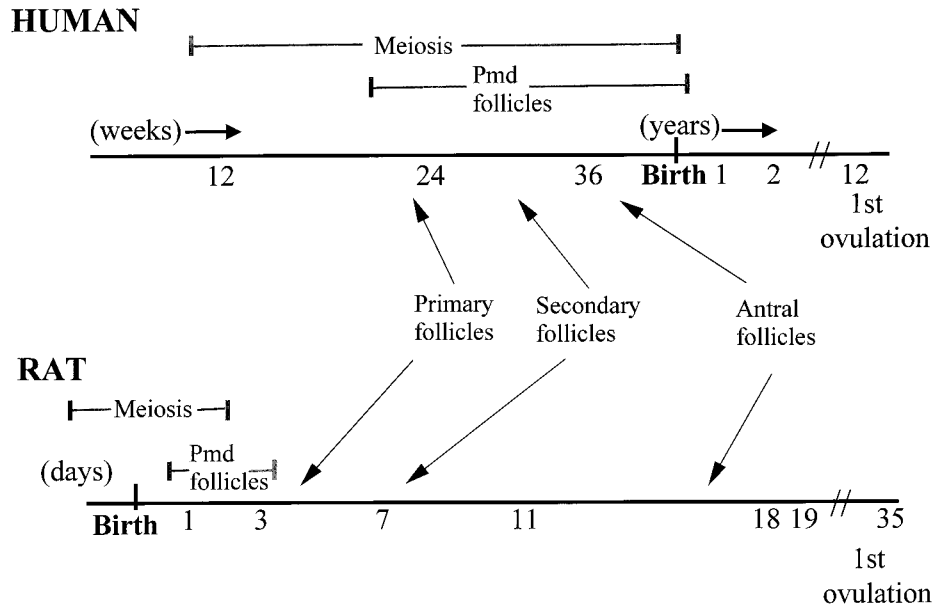

FIG. 3. Landmarks of follicular development during fetal and neonatal life in humans and rodents. In the human ovary, primordial follicles are present by 20 weeks of fetal life, whereas primary follicles are found by 24 weeks. By 26 weeks, some follicles have progressed to the secondary stage. Antral follicles develop in the third trimester and are also seen postnatally when FSH levels are elevated. After puberty, cyclic increases in serum gonadotropins stimulate the antral follicles to become preovulatory follicles during each menstrual cycle. In the rat ovary, primordial follicles are formed by 3 days after birth when the first wave of follicles begins growth. These follicles progress to the early antral stage during the third week of life when serum gonadotropin levels are elevated. After early antral follicles are formed, ovarian cell apoptosis increases. FSH receptors are found by day 7 of age, when secondary follicles are present, followed closely by the formation of LH receptors in the thecal cells. Cyclic ovarian function begins around day 35 of age. The neonatal rodent model allows analysis of early follicle development in a synchronized population of growing follicles. Pmd follicles, Primordial follicles.

mated that more than 120 days are needed for primary follicles to grow into the secondary stage and even longer for the development of primordial follicles into primary follicles (1, 42). Thus, the entire growth phase of a follicle is much greater than 220 days or eight menstrual cycles.

At the time of puberty there are an average of 200,000 follicles remaining in the ovary (43). During reproductive life, continuing growth of primordial and primary follicles into secondary and larger follicles leads to a gradual decrease in the original follicle pool. In addition, the primordial follicle pool could also be decreased due to apoptosis of resting follicles. More than 10 yr before menopause, concomitant with subtle increases in serum FSH and decreases in circulating inhibins, increasing percentages of follicles are lost from the resting pool (44–47). The diminishing follicle reserve serves as a ticking clock to time the onset of menopause. As the result of ovarian follicle exhaustion, menopause occurs at about 51 yr of age, a time point that has been constant for centuries (48). With modern increases in longevity, a significant portion (one-third) of a woman's life is now spent after menopause.

E. Chronicle of early follicle development in rodents

Important landmarks of ovarian development in rats are similar to those in the human; however, the timing is greatly compressed. Primordial germ cells migrate to the gonadal ridge late in embryonic development to become oogonia. At birth, the rat ovary consists of cords and oogonia (Fig. 3). Primordial follicles are formed by day 3 of age, and the first wave of follicles develops into antral follicles over the next 3 weeks (49–53). Well developed secondary follicles are found by day 7 of age. Minimal ovarian cell apoptosis can be found until day 18 when early antral follicles are apparent (54, 55). Puberty or first estrus occurs around day 34. The regular estrous cycles continue until around 10–12 months of age when the cycles become prolonged and irregular (56, 57). By age 12–15 months, animals enter persistent estrus, and this is followed by persistent diestrus and ultimately anestrus (58, 59).

The timing of follicle growth has been meticulously evaluated in adult rats (2). The follicle grows from about 25 μm in diameter (primordial follicles) to 500–800 μm in diameter (preovulatory follicles) over a period of greater than 60 days (or about 15 estrous cycles) (Fig. 2). The time for primordial follicles to grow to the secondary follicle stage may be more than 30 days or comparable to the time (28 plus 2–3 days) to grow from the secondary stage to ovulation. Thus, as in the human, early follicle growth in rodents is very protracted. Of interest, the rate of development of the first wave of follicles in juvenile rats (Fig. 3) is more rapid than that in adult cycling animals (60) (Fig. 2).

II. Initial Recruitment of Follicles and Regulation of Early Follicle Growth

A. Initial recruitment of follicles from the resting pool

Mechanisms controlling the initiation of follicle growth have been difficult to investigate because initial follicle recruitment represents a protracted process characterized by the slow growth of a substantial number of small follicles over a prolonged period of time (49). Most investigators have monitored changes in the number of primordial and/or primary follicles that remain in the ovary at any given time, the supposition being that the decline of follicle numbers in this category is due to follicles leaving the resting pool to enter the growing pool. Because of the difficulties involved in distinguishing between nongrowing and growing follicles, primordial and primary follicles have often been considered a contiguous group (1, 61–63), although primary follicles have been shown to be growing (49).

By counting follicles of different categories in ovarian sections, rates of follicle progression and loss have been estimated (1, 2, 5, 45, 61–63). Despite the limitations of this approach, several useful models have been formulated to describe the dynamics of initial recruitment of follicles. Although a radioactive decay model proposed a constant loss of a fixed number of resting follicles from the original pool over time (61, 64), some studies suggested a decreasing number of follicles initiate growth as the ovary ages, in proportion to the number of remaining follicles in the diminishing supply (65). Morphometric studies further suggested that follicles initiate growth based upon the order in which they were formed (2).

The decay model proposed to account for the baseline rate of the initial recruitment of follicles has been further modified based on observed variations in the rate of follicle loss from the resting pool over the reproductive life span. An accelerated loss of follicles from the resting pool has been found during the initial waves of follicle growth in infantile rodents (66). In addition, morphometric studies have demonstrated that an increasing percentage of follicles is lost in the perimenopausal years in humans (44, 46). In both of these situations, serum gonadotropins are elevated compared with those during the peak reproductive years. The accelerated rate of loss of follicles in infantile rodents has been attributed both to a lack of mature follicles that might exert a negative effect on initial recruitment and to qualitative differences in the first groups of growing follicles (5, 61). Likewise, the accelerated follicle loss in perimenopausal women may reflect intrinsic differences in their remaining follicles. Although the observed follicle depletion may be due to increasing serum gonadotropin levels, changes in gonadotropin secretion could also be the result of diminishing inhibitory influences from a lower number of growing follicles.

B. Role of gonadotropins, intraovarian factors, and the oocyte in primary follicle growth

Resting follicles are likely to be under constant inhibitory influences of systemic and/or local origins to remain dormant (67). A decrease of inhibitory influences and/or an increase of stimulatory factors allow the initiation of follicle growth. In hypophysectomized rodents, decreased initial recruitment of follicles is evidenced by a larger resting pool as compared with nonoperated controls (61, 68). Elevated serum FSH levels are associated with accelerated initial recruitment found in both the early and late stages of reproductive life, as mentioned earlier. High tonic LH/human CG

(hCG) levels may also reduce the number of resting follicles. In transgenic mice overexpressing a long-acting LH, primordial follicles are lost from the resting pool more rapidly than controls (69). However, FSH and LH are unlikely to exert direct actions on primordial follicles because functional gonadotropin receptors have not yet developed in them (70–74). Instead, the ability of FSH to accelerate the development of preantral follicles, as described in the following section, may indirectly increase the loss of resting follicles from the dormant pool. Other factors, such as elevated steroid levels and mediation by paracrine factors, may also have a role in the loss of follicles from the transgenic animals.

Although follicles do not have functional FSH receptors until the secondary stage, pregranulosa cells and primordial follicles respond to activators of the cAMP pathways (*e.g.*, forskolin and cAMP analogs) with increased expression of aromatase and FSH receptors (75). It has been proposed that endogenous activators of cAMP may play a role in the differentiation of follicles after their initial recruitment (76). Treatment of ovarian explants from neonatal rats with vasoactive intestinal peptide or norepinephrine increases cAMP production and accelerates early follicle development. Because the first follicles that grow in the rat ovary are in the highly innervated corticomedullary junction, the first wave of follicle growth may be facilitated by these local neurotransmitters.

The role of the oocyte in the initial recruitment of follicles has been considered. During development, granulosa cells of primordial follicles start to divide (49), followed by morphological changes to the cuboidal shape characteristic of primary follicles. Because an increase in oocyte size is not evident until formation of the primary follicle, a passive role of the oocyte in initial recruitment has been suggested (12). Based on the observation that the number of chiasmata, or crossing-over events, in ovulated oocytes decreases with the increasing age of an animal, it was proposed that the order of follicle recruitment is related to the order in which the oocytes entered meiosis during development (61). This "production line" hypothesis predicts that the first oocytes entering meiosis are the first ones maturing and ovulating. In addition, Hirshfield (2) has demonstrated that rapidly progressing oocytes located near the corticomedullary junction of the ovary begin growth earlier and are enclosed into follicles that initiate growth during the neonatal and infantile period, a time of accelerated follicle loss. In contrast, oocytes that undergo slower meiotic progression are located closer to the cortex and are enclosed in follicles that grow later in life. Meiotic competence of human follicles declines with age (77), and this finding has been used in support of the production line hypothesis. However, the reduction of oocyte quality could be due to poorer conditions present during folliculogenesis after age 35. These issues have yet to be resolved.

Factors involved in oocyte-granulosa cell communication in early follicles have also been proposed to have a role in initial recruitment. The Steel factor or kit ligand is expressed by granulosa cells of growing follicles whereas c-kit, a tyrosine kinase receptor of the platelet-derived growth factor receptor family, is located on oocytes and theca cells. Mutations in mice that prevent the production of the soluble form of the kit ligand lead to failure of follicular growth beyond the primary stage (78–80) (Table 2). Less severe mutations that result in reduced production of the soluble ligand allow a few follicles to grow to the antral stage. These animals ovulate sporadically and show limited fertility. Of interest, treatment of neonatal mice with a neutralizing antibody against the c-kit receptor caused apparent disturbances in initial follicle recruitment, primary follicle growth, and antrum formation in larger follicles (81). Mutations affecting the function of c-kit in humans, however, do not seem to affect female fertility (82). The role of c-kit in human ovarian function requires further study.

Further evidence of the potential role of the oocyte in early follicle development is provided by studies of growth differentiation factor-9 (GDF-9), a homodimeric protein of the transforming growth factor-β (TGFβ)/activin family that presumably signals via serine-threonine kinase receptors. GDF-9 is produced by growing mouse, rat, and human oocytes (83–86) in primary and larger follicles but is absent in primordial follicles. However, in ovine and bovine ovaries, the GDF-9 message could be detected as early as the primordial follicle stage (87). In mutant mice, disruption of the GDF-9 gene prevents follicle development beyond the primary stage (88) associated with an absence of thecal cell markers and eventually oocyte death (89) (Table 2). These studies demonstrated the importance of oocyte-granulosa cell interactions during early stages of follicle development. Because kit ligand and GDF-9 are highly expressed in secondary follicles, they also are likely to play important roles in preantral follicle development. Indeed, recent studies indicated that treatment with recombinant GDF-9 stimulates inhibin-α production by neonatal ovarian explants in rats as well as the growth and differentiation of cultured preantral rat follicles (84). However, GDF-9 production is not obligatory for inhibin production because inhibin-α transcript continues to be found in GDF-9 null mice (89). Based on its sequence homolog to GDF-9 and other TGF-β family proteins, a novel gene, GDF-9B (90), also named as BMP-15 (91), has recently been identified. Of interest, the expression of the

TABLE 2. Mutant mouse models with alterations in preantral follicle development

Gene	Ovarian expression pattern	Phenotype of mutant mice
GDF-9	Growing oocyte	No normal follicle growth beyond the primary stage
Kit ligand	Granulosa cells	Soluble form necessary for follicle growth beyond primary stage
Connexin 37	Oocyte-granulosa gap junction	Defective oocyte/granulosa interaction. Small oocytes not meiotically competent. Antral follicles formed but are small in size
Cyclin D2	Granulosa cells	Reduced number of granulosa cells by secondary stage, small antral follicles

GDF-9B/BMP-15 transcript, as in that for GDF-9, is restricted to the oocyte. It is possible that multiple paracrine factors are involved in the communication between oocyte and somatic cells during early follicle development.

Although these studies provide insight into the growth process of very early follicles, the exact mechanisms propelling the primordial follicles to leave the resting pool remain elusive. Of importance, both kit ligand and GDF-9 are first found in primary follicles and their role in primordial follicle activation remains to be established. Further studies are needed to reveal potential inhibitory factors and/or intraovarian-stimulating factors that are involved in the initial stage of follicle recruitment. Recent establishment of a serum-free culture of baboon primordial follicles capable of initiating growth *in vitro* (92) could provide further insight into the mechanisms of initial follicle recruitment.

C. Preantral follicle growth and differentiation

Compared with the initial recruitment process, substantially more is known about the regulation of subsequent follicle differentiation and growth. After initial recruitment, granulosa cells in primary follicles undergo profound changes, progressively acquiring the differentiated characteristics of epithelial cells found in secondary follicles. The oocyte continues to grow, the zona pellucida is formed, theca condenses around the preantral follicle, and the vascular supply develops.

In vitro studies have shown that granulosa-oocyte communication is essential for normal oocyte growth in early follicles. Immature oocytes separated from granulosa cells do not grow, but oocytes allowed to maintain gap junctions with granulosa cells grow at a near-normal rate (12). In mice, a gap junction protein, connexin 37, is expressed at the oocyte-granulosa cell junction by the time follicles have developed to the secondary stage, whereas follicles of mice that lack connexin 37 do not progress normally (93) (Table 2). These defective follicles contain normal zona pellucida and granulosa cell processes but lack oocyte-granulosa gap junctions and have impaired oocyte-granulosa communication. They progress normally to the late secondary stage and form a limited number of small antral follicles. The oocytes do not reach full size and are not competent to undergo meiosis. Several studies have further demonstrated that oocytes secrete factors to regulate granulosa cell functions (94), including granulosa cell division (95), LH receptor formation (96), and steroidogenesis (97, 98) as well as cumulus cell expansion (85). These studies underscore the concept that granulosa-oocyte communication is important for normal preantral follicle development.

Because preantral follicle development proceeds much slower than that of larger antral follicles, it is possible that ovarian growth and differentiation genes are suppressed during early follicle development. High levels of the Wilms' tumor gene, WT1, are expressed in the granulosa cells of primary follicles in rats with lower levels in secondary follicles and negligible levels in antral and preovulatory follicles (99). Recent studies extend the finding of restricted WT1 expression in immature follicles to diverse avian (chicken) and mammalian (porcine and monkey) species and over the reproductive life span in rats (100). WT1, a transcription factor with zinc finger domains (101), suppresses the expression of several growth factors and their receptors in different cell types. Furthermore, *in vitro* studies demonstrated that WT1 represses activities of the promoters for inhibin-α and FSH receptor, marker genes essential for follicle development. In gel retardation assays, recombinant WT1 proteins interact directly with consensus DNA sequences in the inhibin-α gene promoter (100). Thus, WT1 may act as a stasis factor on smaller follicles, and falling levels of WT1 allow the progression of early follicle development. Mutant mice with deletion of the WT1 gene die during embryonic development, thus preventing analysis of ovarian follicle development. In human males with inactivating mutations of WT1, gonadal dysgenesis is prominent (102); but detailed analysis of ovarian phenotypes in human females with WT1 mutations (103) remains to be performed. Further studies are needed to elucidate the regulation of WT1 expression in human follicles as well as the interaction of WT1 with other genes involved in follicle development.

Granulosa-theca cell interactions may also have a role in the development of early follicles. Although the role of sex steroids in preantral follicle development is still unclear, a recent study suggested that androgen treatment in intact monkeys increases the number of preantral and small antral follicles up to 1 mm in diameter through androgen receptors (104–106). In cultured mouse preantral follicles, androgen treatment also augments follicle growth (107).

Rat preantral follicles have been shown to secrete proteins that enhance the growth and differentiation of theca cells before their expression of LH receptors (51). Likewise, cocultures of theca and granulosa cells enhance proliferation and steroidogenesis of both cell types (108). The observed interactions between granulosa and theca cells are probably mediated by paracrine growth factors. Recent studies indicated that keratinocyte growth factor, or fibroblast growth factor-7, a paracrine hormone secreted by theca cells (109), enhances the growth of preantral rat follicles in culture (110).

Treatment of dissociated ovarian cells from juvenile rats with activin and FSH enhances formation and growth of follicular structure (111). Activin treatment also enhances FSH-stimulated inhibin production in dispersed ovarian cells from neonatal rats (112). In mice, cultured preantral follicles secrete activin, and treatment with recombinant activin enhances FSH-stimulated inhibin and estrogen production (113). Furthermore, studies using cocultures of mouse follicles at different stages of development suggested that activin secreted from secondary follicles causes small preantral follicles to remain dormant (114, 115). The exact stage-dependent effects of activin in early follicle development remain to be elucidated.

High levels of IGF-I and IGF receptors have been found in postnatal rats during preantral follicle development (116). However, follicles seem to develop relatively normally to the early antral stage in mutant mice lacking IGF-I, although numerical morphometrics were not performed (117). Studies using these mutant mice further suggested that ovarian IGF-I expression serves to enhance granulosa cell FSH responsiveness by augmenting FSH receptor expression (118). A large body of data exists on the effects of growth factors on mono-

layer cultures of granulosa and theca-interstitial cells as well as on cultures of antral and preovulatory follicles (5, 24, 119, 120). It is clear that paracrine growth factors are also involved in preantral follicle development. With recent advances in transgenic technology, more than 30 mouse models with ovarian defects at different stages of follicle development have been described (reviewed in Ref. 121). Derivation of additional mutant mice with ovarian phenotypes will further enhance our understanding of early follicle development.

D. Preantral follicles are gonadotropin responsive

Several recent studies investigated the role of FSH on follicle development based on mutant mice with a defective FSH-β or FSH receptor gene (122, 123) and in patients with loss-of-function FSH receptors (124). Although the growth of preantral follicles has been considered to be gonadotropin independent because follicles can develop to the antral stage in animals or humans with minimal circulating FSH or defective FSH receptors (6, 122, 124–127), studies in rodents have suggested that the development of early follicles is under the influence of gonadotropins. During the first 3 weeks of life in rats, the first wave of follicles begins to grow, corresponding to events found during fetal life in the human ovary (Fig. 3). Functional FSH and LH receptors are present when well developed secondary follicles are found by day 7 of age. In addition, serum FSH levels are elevated between days 11 and 19, a time of rapid follicle growth (128–130). Treatment with dihydrotestosterone propionate during the first week of life decreases serum gonadotropins and leads to a delay in ovarian FSH receptor acquisition (131). Conversely, treatment of infantile rats with PMSG increases ovarian weight (132), whereas treatment of neonatal rats with a GnRH antagonist reduces the number of growing ovarian follicles found at puberty (7).

Taking advantage of the relatively uniform development of the first wave of follicles in the postnatal rat ovary, the role of endogenous and exogenous gonadotropins on preantral follicle development was evaluated (50). Reduction of the high levels of gonadotropins present in juvenile rats by either hypophysectomy or GnRH antagonist treatment decreases ovarian weight at day 19 of age and reduces the number of developing follicles together with increasing atresia of the remaining ones. In contrast, treatment with FSH in intact, hypophysectomized, or GnRH antagonist-treated juvenile rats increases ovarian weight and preantral follicle development (50). *In vitro* studies on the role of FSH in preantral follicle development have been conflicting. In one model, FSH treatment promotes the progression of cultured rat follicles to the antral stage (133). In another study, FSH treatment alone does not enhance granulosa cell division or steroidogenesis (111). In mice, FSH treatment enhances antral formation without increasing granulosa cell numbers in cultured follicles (134) whereas, in cultured hamster follicles, FSH treatment increases granulosa cell division (135). Using a cGMP analog to suppress apoptosis in preantral rat follicles in serum-free cultures, it was demonstrated that FSH treatment increases both follicle size and cell number (54).

Recently, FSH has been shown to stimulate the expression of cyclin D2, a cell cycle protein important in the G_1 phase of cell division. Mice lacking cyclin D2 are infertile, and granulosa cell replication is impaired as early as the secondary follicle stage (136) (Table 2). The paucity of granulosa cells results in the formation of small atypical antral follicles that cannot ovulate properly.

Thus, these results demonstrate that preantral follicles respond to gonadotropins with cell division and differentiation. However, as discussed earlier, follicles can progress to the antral stage in the absence of gonadotropins. In hypogonadal mice (127) or hypophysectomized rats (137), ovarian follicles can develop to the secondary and early antral stages, but more slowly and in fewer numbers. In individuals with hypogonadotropic hypogonadism, treatment with exogenous gonadotropins leads to the development of preovulatory follicles within 2 weeks, suggesting that antral follicles are present and available for cyclic recruitment (138). Clearly, continued development of antral and larger follicles is dependent on the presence of FSH, but gonadotropin responsiveness may occur earlier in follicle development (139) than is widely believed (Fig. 4). This concept is further supported by studies on human early follicles in ovarian xenografts transplanted into the kidney capsule of immunodeficient and hypogonadal mice (140). In this model, FSH was shown to be required for the growth of follicles beyond the two-layer granulosa cell stage. Therefore, gonadotropin fluctuations during the estrous cycle in the rodent may not only advance the development of antral follicles but may also affect smaller growing follicles that are several cycles away from becoming the leading cohort. In humans, exogenous gonadotropins could have an effect on follicle development for several months after a controlled ovarian hyperstimulation cycle, although the exact role of gonadotropins remains to be elucidated.

III. Cyclic Recruitment Of Follicles to Escape from Atresia

A. FSH is the survival factor for antral follicles

Before the onset of puberty, the normal fate of growing follicles is atretic demise. After puberty, stimulation by cyclic gonadotropins allows the survival and continued growth of only a limited number of antral follicles that will reach the preovulatory stage. Morphological and biochemical studies have demonstrated that the demise of both somatic and germ cells in the ovary is mediated by apoptosis (40, 141–143). Although apoptosis can occur at all stages of follicle development, in rodents, the preantral to early antral transition is most susceptible to atresia (2). These early antral follicles are 0.2–0.4 mm in diameter in rats, whereas human follicles with a similar developmental fate are 2–5 mm in diameter and have a well developed antrum. Most studies on follicular cell apoptosis have been performed in rats, but there are a few recent studies of human tissues (144–146).

FSH and LH are important trophic factors for the proliferation and survival of follicular somatic cells and the cyclic recruitment of antral follicles. Suppression of serum gonadotropins after hypophysectomy leads to atresia and apoptosis of developing follicles (147), whereas FSH treatment of cultured early antral follicles prevents the spontaneous onset

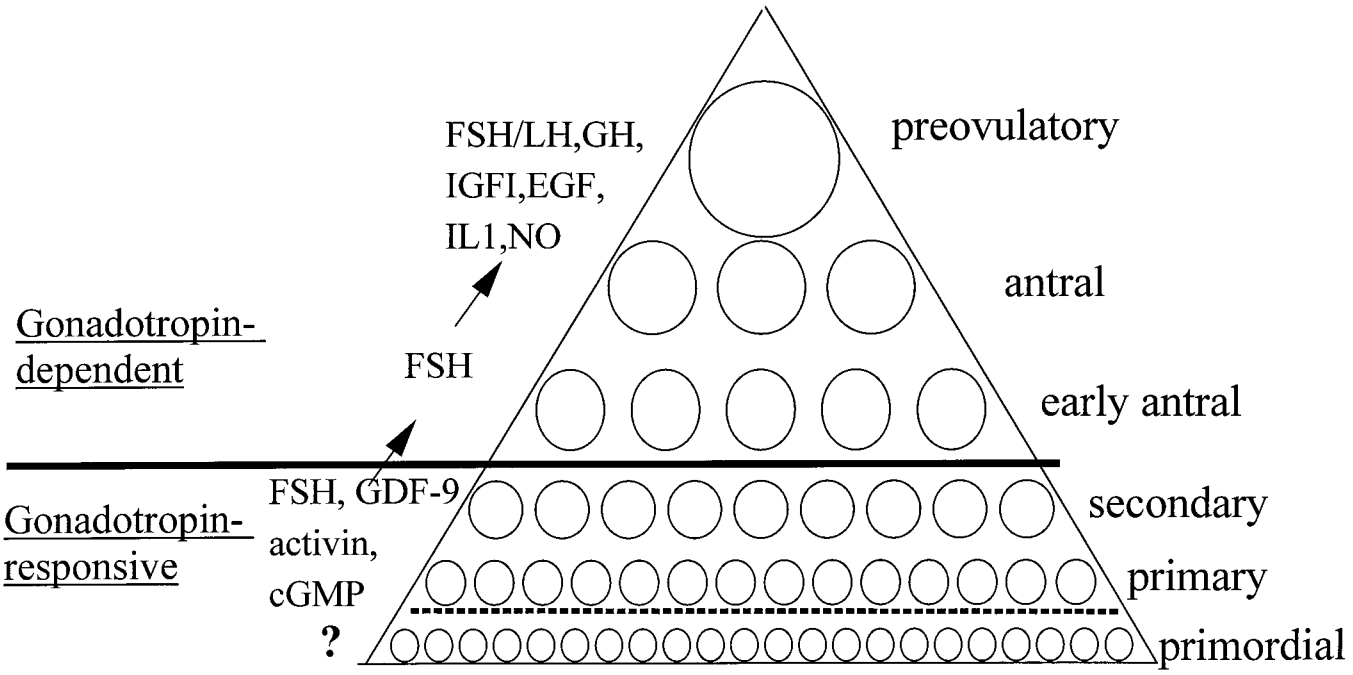

FIG. 4. Follicle mountain: stage-specific hormonal factors are involved in the survival and development of rodent follicles. Studies in rodents indicated that the development of follicles from the smallest primordial and primary follicles to the largest preovulatory follicles requires different stimulatory and survival factors that are stage dependent. Although the exact mechanisms for the initial recruitment of follicles from the dormant primordial follicle pool are still unclear, FSH, activin, and GDF-9 have been shown to stimulate the growth and differentiation of primary and/or secondary follicles. In addition, unknown ligand(s) activating through the cGMP pathway could serve as survival factors for preantral follicles. FSH is the major survival factor to rescue early antral follicles from apoptotic demise during cyclic recruitment. Once the follicles reach antral and larger sizes, multiple intrafollicular factors are produced locally to ensure successful maturation and ovulation. Although it is known that the growth of antral and preovulatory follicles is dependent on adequate gonadotropin stimulation, recent *in vitro* and *in vivo* studies indicated that the growth of preantral follicles could also be enhanced by endogenous and exogenous gonadotropins. Thus, the development of follicles can be divided into gonadotropin-dependent and gonadotropin-responsive stages. During treatment with exogenous gonadotropins, it is likely that both large antral follicles and smaller preantral follicles are stimulated to grow.

of follicular apoptosis (148). However, LH/hCG treatment alone is ineffective, suggesting that FSH is the predominant survival factor at this stage of follicle development (148). In rats, estrogens are potent antiapoptotic hormones in early antral follicles (149), although the role of estrogen in human follicles is still unclear. Follicular estrogen production is dependent upon both FSH stimulation of aromatase in the granulosa cells and LH stimulation of androstenedione production by the theca (24). Therefore, both gonadotropins play a role in the continued survival of growing follicles, but the cellular mechanism by which FSH or estrogen ensures the survival of early antral follicles is unknown.

B. Intrafollicular hormonal mechanisms to ensure survival of preovulatory follicles

It is becoming evident that survival factors are needed to sustain folliculogenesis during the progression of follicle development (Fig. 4). Recent rodent studies indicate that preantral follicles in serum-free cultures undergo apoptosis despite exposure to gonadotropins or cAMP analogs (54), suggesting that gonadotropins are probably not survival factors at early stages of folliculogenesis. As discussed above, FSH seems to be the most important survival factor for early antral follicles. However, in preovulatory follicles, numerous factors promote follicle cell survival (142, 150–152), indicating that overlapping hormonal cascades are involved in maintaining follicles that develop to this stage. The differential responsiveness of follicles to hormonal signals at different developmental stages may ensure a staggered supply of maturing follicles during reproductive life.

An elaborate intrafollicular control mechanism ensures the survival of preovulatory follicles (153). The onset of apoptosis in preovulatory follicles in a serum-free culture is prevented by treatment with FSH and LH (150). In addition, treatment with GH (151) or local factors including IGF-I, epidermal growth factor, TGFα, and fibroblast growth factor-2, likewise suppresses follicle cell apoptosis (150, 154). Interleukin-1β is also a survival factor for preovulatory follicles, and the action of interleukin-1β is mediated through increases in the production of nitric oxide, which, in turn, activates soluble guanyl cyclase (152). Although gonadotropins are the most important survival factors for preovulatory follicles, this array of extracellular signals acting through endocrine, paracrine, autocrine, or juxtacrine mechanisms, ensures their survival for ovulation.

IV. Dynamics of the Follicle Pool: Puzzles and Unanswered Questions

Analysis of the basic mechanisms of follicle recruitment and selection has important clinical implications. Although

the exact mechanisms underlying initial recruitment of follicles is still unclear, hormonal mechanisms underlying cyclic recruitment have been better elucidated. In the following section, several puzzling clinical issues related to altered follicle development and menopausal timing are discussed. Menopause is linked to the exhaustion of the primordial follicle reserve. Because all primordial follicles initiated to grow are programmed to undergo apoptosis unless rescued by FSH, depletion of the follicle pool results from initial, but not cyclic, recruitment of follicles (Fig. 1). However, some factors that are well known regulators of cyclic recruitment may also affect the less characterized process of initial recruitment. Much of the current human data are based on epidemiological analysis. Although these studies can provide valuable clues regarding the mechanisms of altered physiological processes, they are sometimes complicated by compounding variables.

A. Does early onset of menarche lead to a corresponding younger age at menopause?

No. Despite a substantial decrease in the age of menarche in women during the last century, a corresponding change of the menopausal age has not been detected (48). As discussed, initial follicle recruitment and follicle loss from the resting pool begins long before pubertal onset. Changes in the age of menarche as the result of environmental, nutritional, or pathophysiological factors (such as in women with central precocious puberty) allow an earlier onset of cyclic follicle recruitment but should not affect the timing of follicle pool depletion.

B. Do women of reproductive age who have undergone unilateral ovariectomy or chemotherapy have an earlier onset of menopause?

Yes, but it is dependent on the timing of the procedure. Unilateral oophorectomy or chemotherapy, which reduces the pool of resting follicles, shortens the reproductive life span. Although removal of up to 95% of ovarian tissue in monkeys does not affect subsequent reproductive cycles for up to 1 yr (155), the size of the resting follicle pool is a major determinant of ovarian senescence. Thus, unilateral oophorectomy can accelerate reproductive aging. If unilateral oophorectomy is performed in the later part of the human reproductive years when the resting pool is smaller, substantial advancement in menopausal age occurs (45, 46). However, when the same procedure is performed early in life, the menopausal age is less affected, suggesting that a compensatory mechanism in initial follicle recruitment might allow a lower number of follicles to initiate growth (156).

In rodent studies, removal of one ovary accelerates follicle loss in the remaining ovary (30), but halving the total follicle number only results in the loss of a quarter of expected cycles (157). Furthermore, unilateral oophorectomy increases the loss of remaining primordial follicles in middle-aged rats but not in young rats operated on at 30 days of age (158). Likewise, treatment with a chemotherapy agent, busulfan, decreases the resting follicle pool by more than 90% in rats and substantially accelerates follicle depletion (159). Conversely, mutant mice deficient in a proapoptotic gene, Bax, maintain a larger resting pool of follicles later into adult life (160).

In humans, exposure to alkylating agents is associated with premature ovarian failure (161), and the degree of ovarian dysfunction seems to be inversely related to the age of drug exposure (162). Some reports suggest that treatment with GnRH agonist protects against chemotherapy-induced ovarian damage, possibly by reducing the rate of primordial follicle attrition (163, 164). However, recent studies do not show a protective effect of GnRH agonist treatment on radiation-induced follicular injury (165). Given the increased survival rates of patients from cancers of childhood and early adulthood, more studies on potential protective measures of the follicle pool are greatly needed.

C. Do women who have used steroidal contraceptives have delayed menopause?

Probably no. Prolonged exposure to steroidal contraceptive pills mainly affects the ovulatory surges of circulating gonadotropins during the fertile period. Thus, ovulation is suppressed, but follicles continue to grow to the antral stage (6). It would seem likely that neither initial nor cyclic recruitment of follicles would be affected. However, since the advent of the oral contraceptive pill in the 1950s, the first generation of pill takers have reached menopause, and epidemiological studies have suggested that menopausal age may be slightly delayed (166, 167). Further studies of subsequent generations of pill takers are needed to rule out compounding variables in the population of "pill pioneers" (167).

D. Do women with increased parity have delayed menopause?

Yes. Epidemiological studies indicate that women with increased parity show a delay in menopausal onset (166, 168, 169). Prolonged elevation of circulating progesterone during pregnancy may suppress initial follicle recruitment, thus maintaining a larger follicle pool size. Early studies in pregnant mice indicated that fewer follicles start growth per unit time (170). Indeed, middle-aged rats treated with progesterone implants or allowed to undergo multiple pregnancies show a delay in reproductive aging (171). Furthermore, prolonged treatment of young rats with a progesterone implant is associated with the conservation of follicle reserve in aging animals (172). One is unable to distinguish, however, between a direct action of progesterone on resting follicles and an indirect effect of progesterone mediated by changes in gonadotropin secretion. Additional pregnancy-related factors may also override the facilitatory effect of hCG on follicle exhaustion (69) and could result in the protection of the follicle pool. It would be interesting to determine whether users of levenorgestrel implants or injectable medroxyprogesterone acetate showed any change in menopausal timing.

E. Do women with dizygotic twins have an earlier onset of menopause?

Maybe not. The exact mechanism of dizygotic twining is still uncertain, although increased numbers of large antral

follicles have been found in mothers of dizygotic twins during the follicular phase of their cycle (173). Assuming the increases in preovulatory follicles found in these individuals are due to elevated gonadotropins during the early follicular phase of their menstrual cycle (174–176), this condition is likely due to enhanced cyclic recruitment of follicles and should not alter initial recruitment and menopausal age. However, some epidemiological studies have suggested that mothers of twins have an earlier menopause (177, 178), although this may be related to other variables such as cigarette smoking which was not evaluated. Also, women who are carriers of the Fragile X syndrome show increased incidence of dizygotic twinning and an earlier onset of menopause (179). The relationship between the defective FMR-1 (Fragile X Mental Retardation) protein found in these patients, twinning, and ovarian senescence awaits further analysis.

F. Do women who have undergone repeated cycles of controlled ovarian hyperstimulation with gonadotropins have an earlier onset of menopause?

A qualified no because exogenous gonadotropins are believed to act mainly on antral follicles to start cyclic recruitment. Although an accelerated decrease of the follicle pool size before menopause is associated with increases in circulating FSH, it is still unclear whether the premenopausal increase in FSH is the result or the cause of follicle pool depletion. There is no doubt that initial recruitment of follicles can proceed without gonadotropins, but it is unclear whether repeated exogenous gonadotropin treatment might accelerate follicle loss by an indirect action mediated through gonadotropin-responsive preantral follicles. Due to the possibility of underlying ovarian disorders in women who undergo gonadotropin stimulation for infertility, analysis of this issue may be difficult. Women who are egg donors would be an interesting group to evaluate in this regard.

V. Conclusions

Advances in ovarian endocrinology, molecular biology, and transgenic technology provide new perspectives on factors controlling the life history of ovarian follicles and new tools with which to investigate normal and abnormal follicle development. Understanding the hormonal and molecular mechanisms of the initial and cyclic recruitment of follicles, as well as the factors regulating follicle maturation, atretic degeneration, and ovulation, will facilitate 1) the design of new contraceptives, 2) the refinement of culture conditions for the generation of fertilizable eggs (11), and 3) the manipulation of the female reproductive life span and treatment of infertility associated with abnormal follicle development. It is becoming evident that the design of noninvasive methods to monitor the pool size of primordial and primary follicles could be useful in determining the follicular reserve (180). Elucidation of factors involved in the initial recruitment of follicles could provide new treatments for patients with premature ovarian failure. The possibility of suppressing initial recruitment and preventing the growth of resting follicles could be the basis for designing treatments that would preserve the resting follicle pool, thus extending the female fertile period and delaying menopause. Although prolonging cyclic ovarian function minimizes the need for hormonal replacement therapy, age-related increases in chromosomal defects in the oocyte should be considered. Future advances in the elucidation of the regulatory mechanisms underlying cyclic recruitment are essential for understanding the pathophysiology of polycystic ovarian syndrome (181). The combination of basic and clinical approaches to issues associated with follicle dynamics will facilitate the understanding of mechanisms of follicle development and improve clinical practice.

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