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REGULATION OF INHIBIN SYNTHESIS IN THE RAT OVARY

Teresa K. Woodruff and Kelly E. Mayo

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208

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INTRODUCTION

The fundamental structure of the mammalian ovary is the follicle. Cells of the follicle, principally granulosa and theca cells, produce hormones and provide information necessary for the onset of puberty, for control of the reproductive cycle, and for the maintenance of pregnancy. Complex interactions involving pituitary hormones, such as follicle-stimulating hormone (FSH), gonadal steroids, such as estradiol, and gonadal polypeptides, such as inhibin, are required to support development of the rapidly proliferating follicle and to coordinate signaling pathways within the reproductive axis (13, 20, 56). Inappropriate alterations in hormone synthesis or secretion within the hypothalamic-pituitary-ovarian axis result in anovulatory, senescent, or disease states (17, 39, 70).

Inhibin has been recognized for some time to be a key hormone for the appropriate regulation of pituitary FSH secretion (14, 32, 42, 66, 67), although its biological role in reproductive processes is not completely understood. With recent advances in the characterization of the inhibin proteins and their genes, questions regarding the expression and regulation of ovarian inhibin during the reproductive lifespan of the animal are now being investigated. The development of the inhibin concept, the characterization of the inhibin protein, and the description of its biological activities have all been

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the subject of recent reviews (13, 15, 35, 68, 74, 84). This review will focus on the regulation of inhibin production in the female rat during the estrous cycle and during pregnancy. Before examining these endocrine states, we will briefly consider the characteristics of the inhibin family of hormones and their expression in gonadal and extragonadal tissues.

STRUCTURE AND EXPRESSION OF RAT INHIBIN

Structure and Function of Inhibin and Related Hormones

Inhibin is a disulfide-linked heterodimer, consisting of an α chain and one of two highly homologous β chains designated β_A and β_B (36, 49, 58, 61). The α chain is an 18-kd peptide and the β chains are 14-kd peptides, which lead to the formation of a 32-kd α-β dimer in most species. Inhibin A (α-β_A) and inhibin B (α-β_B) (see reference 5 for nomenclature) act on the anterior pituitary to selectively suppress FSH biosynthesis and release (8, 24, 44, 86). A second gonadal hormone, activin, is formed by the dimerization of the inhibin β chains (37, 75). Activin A (β_A-β_A) and activin AB (β_A-β_B) stimulate FSH synthesis and secretion from anterior pituitary cells in culture and act as functional antagonists to inhibin. Combinatorial assembly of the α, β_A, and β_B chains therefore generates multiple hormonal activities able to regulate the pituitary-gonadal axis (73). Activin also has biological activities outside the reproductive system; these will be considered in the following section.

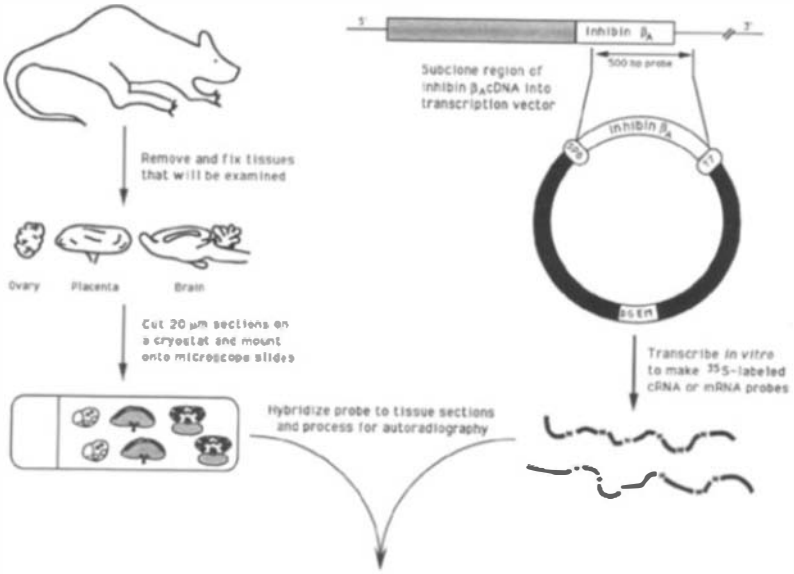
Molecular cloning of inhibin α and β subunit cDNAs from several mammalian species indicates that each of the protein subunits is encoded by a distinct gene, that these genes and the proteins they encode are structurally related to one another, and that the mature inhibin chains reside at the carboxyl-terminus of much larger precursor proteins that undergo proteolytic processing (22, 26, 38, 41, 82). Analysis of inhibin cDNA clones also reveals a surprising structural similarity between the β chains of inhibin (activin) and transforming growth factor-β (TGFβ), a molecule with diverse effects on cellular proliferation and differentiation (38, 40). Like activin, TGFβ can stimulate the secretion of FSH from anterior pituitary cells in culture (85). TGFβ and activin represent early examples of an emerging gene family, which also include mammalian Mullerian inhibiting substance genes (9), the *Drosophila* pattern formation gene decapentaplegic (53), the *Xenopus* gene *VG1*, which encodes a protein involved in mesodermal induction (77), and three related human genes encoding bone morphogens BMP-2, BMP-2A, and BMP-3 (83). This suggests that the hormones of the inhibin family, particularly the activins, may play a role in differentiation and development unrelated to their reproductive function.

Gonadal and Extragonadal Expression of Inhibin Genes

Inhibin was originally isolated from follicular fluid, and the gonads are clearly the primary source of circulating inhibin. Ovariectomy dramatically decreases serum inhibin as measured by bioassay or radioimmunoassay (33, 57, 62), and inhibin bioactivity and protein have been localized to the ovarian granulosa cell (2, 21, 45). All of the inhibin mRNAs are expressed in the ovary, although there is about 10-fold more α than β_A or β_B mRNA (38, 82). Bicsak & coworkers have used biosynthetic labeling of cultured granulosa cells to show that the excess α-subunit protein exists predominantly in the precursor form and is stored intracellulary in addition to being secreted (2). The observed excess in α-subunit expression suggests that the β subunit is likely to be rate-limiting for inhibin production, and that α-β heterodimers are likely to predominate over β-β dimers in the ovary. In the male, the inhibin mRNAs are also predominantly expressed in the testes, although testicular expression declines following the onset of spermatogenesis (24a, 31, 47). Inhibin is made by the testicular sertoli cells (45, 50), while activin is secreted by interstitial cells (34). Although this review will focus on the endocrine actions of inhibin, both inhibin and activin may also have paracrine functions in intragonadal signaling (30).

Because the ovary is a complex tissue, in situ hybridization has been a powerful tool for examining inhibin gene expression at the cellular level. This technique is outlined briefly in Figure 1, which shows several examples of inhibin β_A mRNA localization in both gonadal (ovary) and extragonadal (placenta and brain) tissues. In the ovary, the inhibin mRNAs are predominantly localized to the granulosa cell layer of healthy developing follicles, although the α mRNA is expressed at low levels elsewhere (46, 79). Ovarian expression will be considered in greater detail in a subsequent section.

A comprehensive survey of rat tissues by RNA hybridization has been reported by Meunier & coworkers (47). They observed that the α, β_A, and β_B mRNAs were expressed in a variety of tissues at differing ratios. The finding that the inhibin subunit mRNAs are widely expressed in nonreproductive tissues is intriguing and is consistent with roles for the inhibin and activin proteins outside the reproductive axis. Inhibin and activin expression has been further examined in several nongonadal tissues including the brain, pituitary, bone marrow, placenta, and adrenal. In the brain, inhibin α, β_A and β_B mRNAs were all detected by solution hybridization (47). The β_A subunit has been immunologically localized to neuronal fibers and cell bodies in the caudal medulla (64). The data shown in Figure 1 suggest that the protein is synthesized in this region, in that cell bodies predominantly localized in the nucleus of the solitary tract express the β_A mRNA. Some of these β_A-



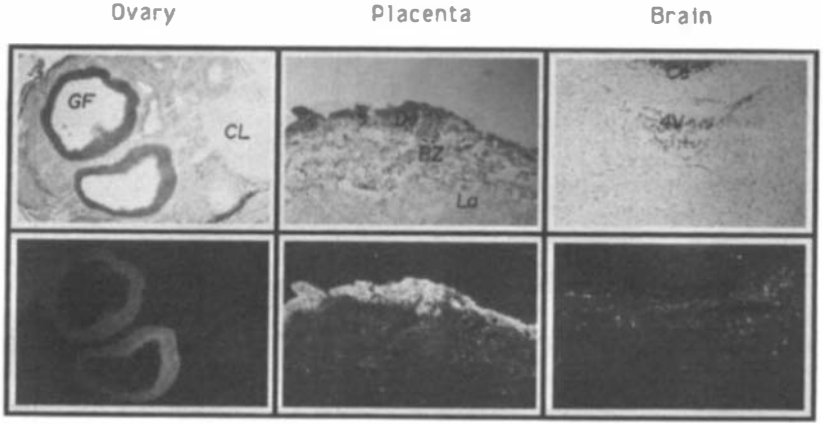


Figure 1 Localization of inhibin β_A-subunit mRNA in diverse cells types using in situ hybridization. The upper portion of the figure shows a schematic diagram of how tissues and probe are prepared for hybridization. The bottom portion of the figure shows localization of the β_A mRNA in ovary, placenta, and brain. The upper photograph in each case is a brightfield photomicrograph of a hemotoxylin-eosin (ovary and placenta) or cresyl violet (brain) stained section. The bottom photograph in each case is an identical field viewed under darkfield optics so that the silver grains indicative of inhibin β_A hybridization can be observed. Hybridization is seen in a mature ovarian follicle from a proestrous animal, in the decidual layer of the term placenta, and in neuronal cell bodies located predominantly in the nucleus of the solitary tract. Abbreviations are GC= Graafian follicle, CL= corpus luteum, De= decidua, BZ= basal zone, La= labyrinth, Ce= cerebellum, and 4V= fourth ventricle.

producing neurons send projections to hypothalamic magnocellular regions involved in oxytocin synthesis and therefore may participate in the modulation of oxytocin secretion (64). The α-subunit protein does not appear to be expressed in these cell groups.

The pituitary is also a site of inhibin or activin expression. The α-and β_B-subunit mRNAs, but not the β_A mRNA, are found in pituitary, and the α and β_B proteins have been localized to gonadotropes using immunocytochemistry. The expression of these subunits in the pituitary is influenced by estrogen (60). Since the anterior pituitary represents the major target for inhibin action, the presence of the α and β_B subunits in gonadotropes suggests possible paracrine or autocrine roles for inhibin or activin in regulating pituitary function. The inhibin α-subunit mRNA is also quite highly expressed in the adrenal cortex, thus suggesting a possible role in the regulation of corticosteroid production (10).

One of the more unexpected sources of extragonadal expression of an inhibin gene is in the bone marrow. Bone marrow appears to express only the β_A mRNA and is therefore likely to produce activin (47). Eto & coworkers have described the characterization of a factor that induces differentiation of a erythroleukemic cell line; this factor was subsequently shown to be identical to activin (23, 51). The biological activities of both highly purified and recombinant inhibin and activin in the hematopoietic system have been examined. Activin has been shown to induce erythroid differentiation, hemoglobin accumulation, and bone marrow colony formation, while inhibin antagonizes this function (4, 64a, 87). The mechanism by which the inhibin subunits exert their broad-ranging biological effects in these tissues has not been fully established, but the emerging evidence suggests that inhibin and activin are active outside the reproductive axis as important regulators of cellular differentiation.

Regulation of Inhibin Production by Granulosa Cells

Granulosa cells are affected by a spectrum of hormonal stimuli and respond based on their maturational status. The regulation of inhibin secretion by steroid and polypeptide hormones has been examined in primary rat granulosa cells by using either hybridization to measure inhibin mRNA within the cells or radioimmunoassay to measure inhibin protein in the culture medium. The levels of secreted inhibin and of inhibin mRNA are positively regulated by FSH in granulosa cell cultures, consistent with in vivo data that indicate that FSH is an important regulator of inhibin gene expression. (3, 69, 82, 86, 89). In addition, FSH directly increases the transcription rates of the inhibin α and β_A genes in primary granulosa cells, as demonstrated by in vitro nuclear run-on transcription assays (T. Woodruff, K. Mayo, unpublished). Luteinizing hormone (LH) stimulates inhibin production only after induction of its

receptor with FSH (3, 72). Pharmacologic agents that act to enhance cellular cAMP levels also stimulate inhibin production by granulosa cells, which suggests that the effect of FSH and LH on inhibin production is cAMP-mediated (3, 69).

Many other peptide hormones modulate inhibin production by granulosa cells. Gonadotropin-releasing hormone (GnRH) attenuates the inducing effects of FSH on inhibin α mRNA accumulation and inhibits FSH-stimulated inhibin secretion in this system (3, 82), consistent with its actions in inhibiting granulosa cell differentiation (29). Vasoactive intestinal peptide, somatomedin C, insulin, and TGFβ have all been reported to increase inhibin production, while epidermal growth factor appears to be a negative regulator (3, 69, 88, 89). Steroid hormones by themselves seem to have little effect on inhibin secretion in this system, although androgens and corticosteroids have been reported to modulate FSH-induced inhibin secretion (69, 72).

REGULATION OF OVARIAN INHIBIN AS A FUNCTION OF REPRODUCTIVE STATUS

Inhibin Expression and Follicular Development During the Estrous Cycle

The reproductive cycle of the female rat is characterized by dynamic changes in serum steroid and gonadotropin levels as well as in follicular development. These changes are coordinated, in part, by alterations in the expression of ovarian inhibin (65). Folliculogenesis is a complex process that involves the growth and death of follicles, the formation of corpora lutea, and the appropriate production of steroid and polypeptide hormones by cells of the follicle (56). Inhibin synthesis is modulated as the follicle matures, a process that can be followed by examining inhibin production during the rat estrous cycle using in vitro bioassay (19), radioimmunoassay (28, 59a), or RNA hybridization approaches (46, 79).

The initiation of expression of the inhibin subunit mRNAs in the antral follicle occurs in two steps. On the morning of estrous, follicles are recruited that have the potential to ovulate four days later (the ovulatory pool). The α-subunit mRNA is found at low levels in follicles prior to their recruitment into this ovulatory pool (11, 46). These small antral follicles have previously been selected from the primordial pool to begin development. The initiator of this developmental pathway, as well as the role of independent α-subunit mRNA expression in these follicles, is unknown; perhaps the free α chain serves a paracrine or autocrine function in directing the subsequent ability of the follicle to enter the ovulatory pool. Once follicles have been recruited by the secondary FSH surge on the morning of estrous, these larger follicles (350 μm diameter) express the α-inhibin mRNA at higher levels and also initiate

expression of the inhibin β_A mRNA. The increase in inhibin mRNA observed in newly recruited follicles on the morning of estrous is paralleled by an increase in serum inhibin, which leads eventually to the decline of FSH to basal levels (28). These changes in inhibin gene expression observed on the morning of estrous are likely to be controlled by the secondary FSH surge. This has been demonstrated in unilaterally ovariectomized (ULO) animals, in which there is a transient rise in serum FSH following removal of one ovary that leads to a compensatory follicular recruitment in the remaining ovary (6, 78). This ULO-induced rise in FSH leads to elevated expression of the inhibin mRNAs in these newly recruited follicles within 24 hr of surgery (11).

The follicles that are initially recruited on the morning of estrous do not all continue in the ovulatory tract. Approximately half of the follicles undergo a process of selective degeneration known as atresia (52). The atretic follicles, like the prerecruited follicles, express the α-and β_A-subunit mRNAs divergently (46, 80). Both mRNAs are dramatically reduced in the earliest stage of histologically identifiable atresia; the β_A mRNA is essentially undetectable in these follicles, while the α mRNA remains in low abundance until the later stages of atresia. The divergent expression of the inhibin subunit mRNAs in atretic and prerecruited follicles is intriguing. Firstly, it in part explains the observation that there is a 10-fold excess of α to β_A mRNA in whole ovarian RNA (38, 82), while in mature follicles examined by in situ hybridization the α and β_A mRNAs appear to be expressed at similar levels; the large pool of nonrecruited follicles presumably contributes substantially to the observed excess in α mRNA. Secondly, it suggests that production of the β_A subunit may be rate-limiting for inhibin heterodimer assembly in ovarian follicles. Lastly, it provides evidence for independent regulation of expression of the inhibin α and β_A genes.

Those follicles that escape atresia and continue to develop progressively increase in size (from 350 to 500 μm diameter) and also accumulate high levels of the inhibin mRNAs (46, 79). Likewise, serum inhibin levels rise during this time (28, 59a). Follicular development and enhanced inhibin expression are presumably under the influence of basal serum FSH. In support of this idea, inhibin mRNA levels increase in follicles that are progressing through the estrous cycle when FSH is transiently elevated following unilateral ovariectomy (11). The accumulation of the inhibin mRNAs in follicles of the ovulatory pool reaches a peak late in the afternoon of proestrous in Sprague-Dawley rats maintained on a 14 : 10 light : dark cycle. The reason for this large rise in inhibin levels on proestrus is unknown, but it may be that inhibin plays a role in synchronizing the subsequent preovulatory FSH surge. The preovulatory LH and FSH surges occur during the early evening of proestrous, and this elevation in gonadotropin levels stimulates mature follicles to initiate events that will lead to ovulation and subsequent luteinization.

Following the primary gonadotropin surges, inhibin gene expression is down-regulated, and the inhibin mRNAs are dramatically decreased in stimulated follicles by late in the evening of proestrus. Serum inhibin levels also decline during the proestrous to estrous transition (28, 59a). This decrease in inhibin expression appears to require the primary gonadotropin surges, since inhibin mRNAs remain elevated in mature follicles if the gonadotropin surges are blocked with a GnRH antagonist (57a, 81). This decrease in inhibin is postulated to allow serum FSH to remain elevated into the morning of estrous (secondary FSH surge), which serves to recruit a new crop of follicles that begin to produce inhibin, as outlined above. Meunier & coworkers have observed that in another strain of animals maintained under different lighting conditions the β_A subunit mRNA is turned off as described above, but that expression is reinitiated prior to ovulation, at a time when the α mRNA is not expressed (46). They suggest that activin is formed in these follicles and that it might function to enhance the secondary FSH surge.

Following ovulation and luteinization, inhibin mRNAs remain low in the newly formed corpus luteum. There is no detectable β_A mRNA in the corpus luteum (46, 79, Figure 1), although the α mRNA continues to be expressed at low levels (12, 46). The α-subunit protein has been immunocytochemically localized in the corpus luteum, which suggests that the protein may remain stable there after synthesis has diminished (45, 46).

The primary and secondary FSH surges are clearly independent with respect to the role inhibin plays in their generation. The primary FSH surge is GnRH-dependent, occurs despite elevated inhibin expression, and results in a subsequent decline of inhibin mRNA expression. The secondary FSH surge is GnRH-independent, occurs only when inhibin gene expression is turned off, and results in an enhanced expression of the inhibin subunit mRNAs in newly recruited follicles. Maintenance of the estrous cycle is therefore critically dependent on precise interplay between inhibin production in the ovary and FSH secretion from the pituitary. These relationships between pituitary FSH secretion and ovarian inhibin production at various points in follicular maturation are schematically depicted in Figure 2.

Inhibin Expression During the Fertile Cycle

Pregnancy is a unique endocrine state in which maternal cyclicity is replaced by a relatively quiescent period of gonadotropin secretion and follicular development (27). Maternal hormones in part direct the development of the fetal endocrine system. Moreover, the placenta provides a novel source of hormones that may act on both maternal and fetal targets. The pattern of inhibin expression in the maternal ovary during gestation is similar to that seen in the cyclic ovary; inhibin α, β_A, and β_B mRNAs are localized to granulosa cells of healthy antral follicles and diminish in the atretic follicles

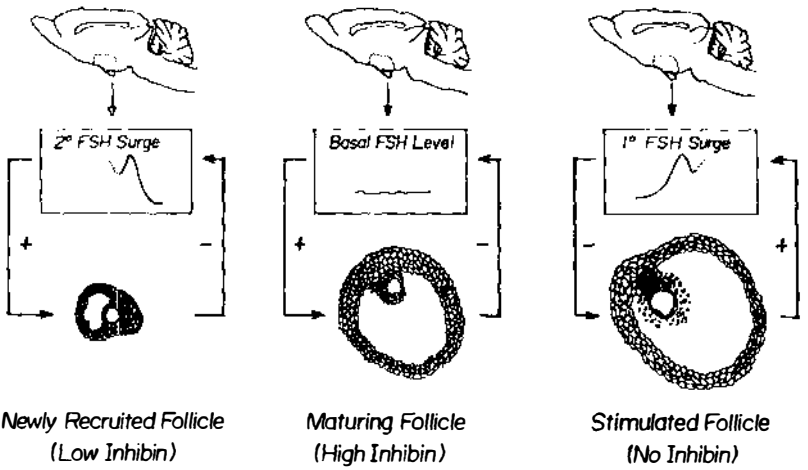


Figure 2 A model illustrating interactions between pituitary FSH secretion and ovarian inhibin production as a function of the maturational status of the follicle. Stimulatory effects are indicated by (+) and inhibitory effects by (–). In early follicular development FSH stimulates inhibin production, but in mature follicles the primary gonadotropin surges signal a rapid decline in inhibin gene expression.

characteristic of early pregnancy. The number of inhibin-expressing follicles is elevated early in pregnancy, falls to a mid-gestational nadir as a result of atresia, and rebounds to increase dramatically in late gestation as follicles that will ovulate following parturition mature (T. Woodruff et al, submitted). Following the postpartum ovulation, few hybridizing follicles are observed. Inhibin activity in ovarian venous plasma mirrors these changes in inhibin mRNA expression (71). Although the corpus luteum becomes functional for progesterone production, no expression of the inhibin mRNAs is observed in the corpora of pregnancy (T. Woodruff et al, submitted).

The fetal-placental unit develops following implantation of the blastocyst on day 5 or 6 of pregnancy in the rat. In addition to secreting hormones important in maternal endocrine regulation, the placenta produces a variety of factors that participate in the establishment of the fetal endocrine system. The role of inhibin in this process is not well understood, but inhibin and/or activin do appear to be placental hormones (1). Although the physiologic role of placental inhibin has not been examined in the rat, human placental extracts can decrease pituitary FSH secretion in a dose-dependent manner (43), and placental inhibin influences the production of chorionic gonadotropin in human placental primary cultures (54). We have localized inhibin α and β_A mRNAs to the cytotrophoblast cells in human placenta; these cells also express GnRH mRNA and peptide (T. K. Woodruff, K. E. Mayo, un-

published; 48), corticotropin-releasing factor (55), and somatostatin (76), thus suggesting that in the human inhibin is produced in cells that participate in directing endocrine aspects of early growth and development. Interestingly, the β_A mRNA is highly expressed in the rat placenta (47) while the α mRNA is nearly undetectable. The β_A mRNA is localized to the maternally-derived decidual cell layer (see Figure 1), which suggests that activin may be secreted from these cells.

Inhibin and the Immature and Senescent Animal

The establishment of adult reproductive cyclicity is preceded by a period of relative pituitary non-responsiveness during which FSH levels rise and fall and profiles of inhibin secretion change. Inhibin is produced in the prepubertal animal, and stimulation of immature animals with pregnant mares' serum gonadotropin results in elevated immunoreactive inhibin and inhibin α mRNA, which suggests that its synthesis is FSH-regulated (12, 33). Rivier & Vale have observed that from days 5 through 20 after birth serum inhibin and FSH levels rise simultaneously, but that at day 21 serum FSH levels decline while serum inhibin levels abruptly rise (59). Sander & coworkers have also demonstrated a progressive increase in the levels of bioactive inhibin in ovarian venous plasma during development (63). The inhibin produced by the immature animal plays a physiologic role in FSH regulation only after day 10. When an inhibin antiserum was infused into immature animals on days 10, 20, and 30 after birth, a significant elevation in serum FSH was found only on days 20 and 30 (59).

As the animal ages, regular estrous cycles are replaced first by irregular cycles and ultimately by an anestrous state. Accompanying these changes are ovarian follicular exhaustion and an impairment in the appropriate regulation of FSH synthesis and release (7). A larger percentage of follicles do not ovulate and continue to produce high levels of estradiol (25). Serum FSH levels become elevated and there is an increased duration of the estrous cycle (18). Moreover, the anterior pituitary becomes less sensitive to the negative influence of inhibin. DePaolo has examined the levels of bioactive inhibin as the adult female ages (16), and found that bioactive inhibin levels progressively decline. Reproductive senescence can be characterized as a deterioration of the influence of ovarian inhibin on the pituitary, which leads to an increase in serum FSH and, ultimately, to follicular exhaustion.

SUMMARY

The lifespan of the female rodent is characterized by dynamic changes in the hormonal regulation of the reproductive axis. From the time ovarian follicular growth is initiated in peripubertal animals, through recruitment, ovulation and luteinization during the estrous cycle, to the quiescent follicular development

of pregnancy, and ultimately to follicular exhaustion, tremendous changes in follicular architecture, hormonal responsiveness, steroid secretion, and inhibin and activin synthesis occur. Similar changes in both the biosynthetic functions and hormonal responsiveness of the pituitary are likely to occur. Some of the information on inhibin expression during the reproductive lifespan of the rat reviewed here is summarized in a schematic fashion in Figure 3, which presents changes in serum FSH, ovarian inhibin production, and follicular development during different reproductive states. From recent observations regarding ovarian inhibin expression as a function of reproductive status, a partial picture of the complex interactions between steroid and peptide hormones necessary to maintain reproductive cyclicity in mammals is beginning to emerge. Study of the inhibin gene family and of other FSH-regulatory hormones is likely to further enhance our understanding of both normal reproductive processes and of reproductive disorders.

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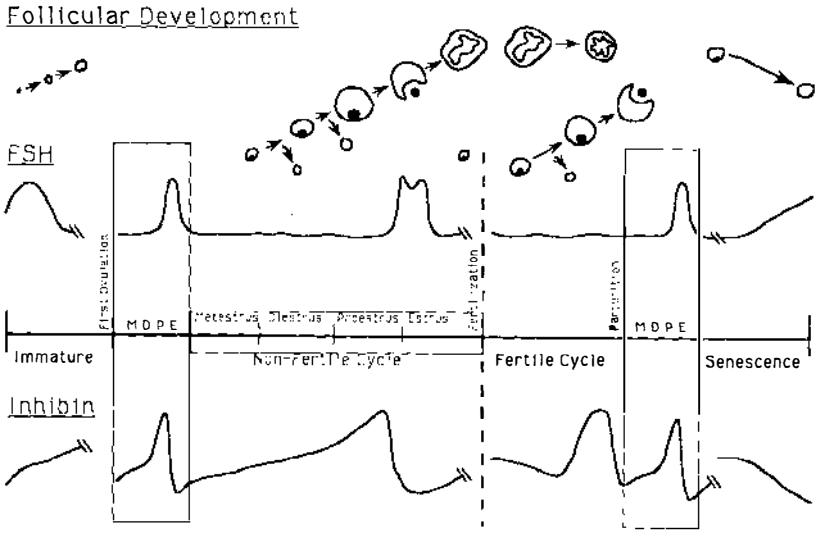

Figure 3 Changes in follicular development, FSH secretion, and inhibin production during the reproductive lifespan of the rat. A central time-line indicates periods of early development, reproductive cyclicity, pregnancy, and senescence. The repeated shaded regions indicate the four day rat estrous cycle, which is expanded in the central portion of the figure. The process of follicular atresia and maturation, ovulation, and luteinization are shown schematically at the top of the figure.

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