CHAPTER

6

Inhibin, Activin, and Follistatin in Ovarian Physiology

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BIOCHEMISTRY AND MOLECULAR BIOLOGY OF INHIBIN, ACTIVIN, AND FOLLISTATIN

In 1923, Mottram and Cramer [1] reported that irradiation of rat testes damaged seminiferous tubules. Interestingly, this treatment also caused the histology of pituitary cells to be radically altered by the appearance of "castration cells" or cells that have secreted the majority of their storage vesicles, suggesting that a factor regulating these pituitary cells comes from testes and is lost due to castration or irradiation. Shortly thereafter, McCullagh [2] reported that an aqueous testicular extract devoid of steroid hormones was sufficient to prevent formation of castration cells, thereby providing evidence for a proteinaceous substance that was secreted from the testis, traveled through the blood and regulated pituitary cell function, that is, an endocrine hormone. He coined the name "inhibin" for this biological activity. This observation set off a 60-year international effort to purify and identify the molecular nature of inhibin.

Further advances required identification of the pituitary gonadotrope hormones that regulate gonadal function in males and females, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and development of assays to measure FSH and LH. With these developments, it became clear that the near complete release of FSH from gonadotropes after gonadectomy produced the "castration cell" appearance. This critical observation provided a reliable bioassay, first in vivo and later, using pituitary cells in culture, that could be used to purify inhibin from gonadal extracts based on its ability to specifically inhibit FSH release without affecting LH [3]. As it turns out, authentic inhibin was finally purified by four different groups in 1985 [4–7] at the time when molecular biology was becoming more widely applied in endocrinology. Thus, the first complete sequence for inhibin was produced not by sequencing the entire protein, which would have required large quantities of pure inhibin, but from its cDNA [8].

This combination of protein purification and molecular sequencing identified several features of inhibin structure. First, inhibin was composed of two disulfide-linked subunits designated alpha and beta. Moreover, inhibin was produced from larger precursor proteins that were proteolytically cleaved to form mature, 32,000 MW inhibin whose sequence and processing were identified from the nucleotide sequence. In this regard, the α-subunit was distinct from the β-subunit in that it had two cleavage sites creating pre-pro, pro, and mature fragments, which produced a stand-alone version of the α-subunit called pro-α-C as well as the mature dimerization partner for β-subunits [9]. Additional β-subunits were identified and named βB, βC, βD, and βE over the years. βA and βB could be linked to α-subunit to form inhibin A and inhibin B, respectively [9]. Finally, the structure and sequence was found to be similar to that of TGFβ, which had been purified and sequenced earlier [8]. This put inhibin within the enlarging TGFβ superfamily that now contains 30–50 members depending on how strictly the families are defined.

Purification of inhibin using the pituitary cell bioassay had some additional advantages. Once the inhibin structure was defined, side fractions from the original purification were reanalyzed. A factor that specifically stimulated FSH release from gonadotropes was identified, named activin, which turned out to be a homodimer of β-subunits, creating activin A (2 βA subunits), activin B (2 βB subunits), and activin AB with one of each [10]. Another side fraction had weaker FSH inhibitory activity that was eventually called follistatin. Follistatin consisted of a single protein chain of approximately 35 kD [11] that was totally unrelated to inhibin or activin. The function of this protein was somewhat mysterious given that a more

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active gonadal FSH release inhibitor (i.e., inhibin) was produced by the same tissues. However, follistatin was determined to inhibit FSH by binding and neutralizing activin [12]. Thus, activin stimulated FSH production and release while follistatin inhibited this action by binding and neutralizing activin, establishing follistatin as a paracrine/autocrine regulator of this new feedback system. The final protein in this story is follistatin like-3 (FSTL3, aka follistatin-related gene; follistatin-related protein), which was discovered in the mid-1990s based on sequence similarity to follistatin [13]. FSTL3 is structurally very close to follistatin suggesting that they originated from a common ancestor, but have different production profiles and activities [14]. With respect to ovarian physiology, FSTL3 does not appear to have important actions [15] and will therefore not be discussed further.

The last decade of the 20th century and the first of the 21st saw a virtual explosion of biochemical, molecular, and physiological studies defining important details of production and action of inhibin, activin, and follistatin in regulating reproduction in males and females. In addition, development of assays, first a radioimmunoassay [16] followed by more specific two-site ELISA assays, allowed investigation of inhibin, activin, and eventually follistatin in animal and human fluids [17]. Most of this work has been compiled and reviewed in a number of scholarly compilations (e.g., Ref. [18]). Moreover, most of the recent advances in this field have been in translation of the basic findings to clinical observations and applications. Therefore, we aim to provide an update from the earlier work that is the basis for current and future clinical applications. It should also be kept in mind that these proteins have actions outside of reproduction that might also be clinically important but won't be covered here.

STRUCTURAL FEATURES, SIGNALING, AND REGULATION OF INHIBIN, ACTIVIN, AND FOLLISTATIN ACTIVITY

As mentioned above, both inhibin α-subunit, and the inhibin/activin β-subunits are produced as pro-protein precursors that are cleaved by furin proteases to produce mature, dimeric proteins [9]. While the activins are inactive until the propeptide is cleaved, bioactive inhibins of various sizes have been identified containing unprocessed or partially processed α- or β-subunits in the inhibin dimer [19]. Whether these inhibin isoforms have different activities or just collectively comprise the "inhibin" signal remains to be determined. However, the most N-terminal fragment of the α-subunit precursor is secreted separately from mature inhibin and was recently found to regulate inhibin activity by binding to the site where mature α-subunit binds to the betaglycan inhibin receptor [20] although the physiological significance of this observation is unknown.

Since cleavage of the propeptide from activin is required for its activity it has been assumed that this portion of the molecule is required only for proper dimerization within the endoplasmic reticulum. However, other TGFβ superfamily members are secreted as complexes of the mature and propeptides that are cleaved but still inactive. An example is TGFβ itself, which is activated by proteolytic release from the propeptide at its site of action [21]. The activin propeptide was found to associate with mature activin but the affinity of this interaction was too low to neutralize activin actions as in the TGFβ situation [22]. By engineering a propeptide sequence that matched TGFβ in the C-terminal region but matched activin A in the N-terminal region, the affinity of this propeptide was vastly increased to the point that it became a specific antagonist for activins A or B [23] although still less potent but more specific for activin than follistatin. This propeptide can therefore be used to specifically inhibit activin activity in vivo [24].

In addition to sharing an overall molecular structure, members of the TGFβ superfamily also share a family of receptors for transducing their signals and a family of second messengers for transmitting signals to subcellular compartments including the nucleus. Activins signal through a heterotetrameric complex comprised of one of two ligand-binding type 2 receptors, ActRIIA or B (ACVR2A or B, respectively) that induce a conformational change in activin that opens up binding sites for the signal-inducing ActR1B (Alk4 or ACVR1B) receptors (reviewed in Ref. [18]). The inhibin receptor consists of ActRIIA or B in association with the betaglycan co-receptor with contacts to ActRII being conducted by the inhibin β-subunit while contacts with betaglycan are mediated by the α-subunit [25,26]. Thus, inhibin can act, at least in part, by binding to and therefore sequestering type II activin receptors that are then no longer available for activin signaling. Follistatin and FSTL3 both inhibit activins A and B [14,27,28]) by forming a complex of two follistatin molecules arranged head to tail around the activin dimer [29,30], an arrangement that covers up receptor binding regions on the ligand as well as creating a stable complex with a very slow off rate [27]. Therefore, both follistatin and FSTL3 are relatively high affinity antagonists of activins A and B.

Once activin associates with its receptor complex a conformational change is induced that results in phosphorylation of the ActR1B receptor, which then phosphorylates the second messengers Smad2 or Smad3 [31]. These same Smads are also used by TGFβ itself acting via TGFBR1(ALK5) and it remains unknown how these signals are differentiated at the DNA level from activin-stimulated signaling. Nevertheless, activated

Smad2 or 3 binds with a common Smad4 and the complex then migrates to the nucleus where it induces alterations in gene transcription.

SYNTHESIS AND ACTION OF INHIBIN, ACTIVIN, AND FOLLISTATIN IN THE OVARY

The primary source of inhibin is gonadal since it rapidly becomes undetectable after castration [32]. Activins, although also produced in gonads, are produced in many other tissues so that the source(s) of circulating activin remains enigmatic. Moreover, activin may be more relevant as an autocrine/paracrine modulator of cellular physiology rather than an endocrine hormone, which has made elucidating its biological actions more challenging [31]. Similarly, serum levels of follistatin are unaffected by gonadal status [33] suggesting that locally produced follistatin within the ovary may have more critical actions regulating activin-induced ovarian development and follicular maturation. The primary action of follistatin remains binding and neutralizing activin and related TGFβ family ligands [34] so that the role of activin and follistatin in ovarian development and physiology is tightly intertwined [18].

Although FSH has been known to stimulate secretion of inhibin A and B from ovarian granulosa cells, the differential regulation of inhibin A and B during the human menstrual cycle, taken in conjunction with FSH secretory patterns suggests that this regulation is more complicated (reviewed in Ref. [18]). Inhibin B increases during the luteal-follicular transition as one menstrual cycle ends and the subsequent one begins. It rises to a peak in the mid-follicular phase, with a second peak on the day after the LH surge. Inhibin A on the other hand begins to rise late in the follicular phase reaching one peak at mid-cycle and another during the mid-luteal phase. This suggests that regulation of inhibin A and B production and secretion is not identical nor is their activity at the pituitary [18]. While purified inhibin A and B both suppress FSH in pituitary cell bioassays in vitro [9] the relative roles of inhibins A and B have yet to be established in the human. Further, inhibin overlaps with estradiol in regulating FSH production. Therefore, the relative role of estradiol vs. the inhibins has also been difficult to determine (discussed further below).

The distinct secretory patterns of inhibins A and B may be regulated, at least in part, by the maturational stage of granulosa cell development. In vivo, FSH administration stimulates both inhibin A and B secretion in the early follicular phase from small antral follicles. In vitro, cultured granulosa cells from this stage secrete inhibin A in response to both FSH and cAMP but not inhibin B [35], as well as in response to FSH or LH treatment in vivo [36,37]. The inhibin β promoter does not have a cAMP response element which might explain this lack of direct stimulation [38,39]. Several studies suggest that other TGFβ family members, such as activins, BMPs and TGFβ contribute to upregulating inhibin βB subunit production and thus, inhibin B production and secretion [40,41]. In fact, early in the follicular phase when inhibin B rises, an expanding cohort of small antral follicles is growing in response to FSH, suggesting that increased follicle number and their contained expanding and maturing granulosa cells accounts for increased inhibin B levels during the follicular phase in concurrence with FSH. Inhibin B then suppresses FSH via negative feedback at the pituitary, resulting in selection of a single dominant follicle each cycle [18].

Inhibin α and inhibin/activin βA and βB subunit mRNAs and proteins are expressed within granulosa cells at various stages of development. The regulation of α-β dimerization to form inhibin versus β-subunits dimerizing to form activins A, B, or AB remains incompletely understood. One critical factor is the overall level of α-subunit synthesis which is regulated largely by cAMP since increased α-subunit production favors α-β dimers of inhibin [9,42]. In addition, mature α-subunit is glycosylated and elimination of this carbohydrate chain inhibited inhibin secretion without altering activin while mutating β-subunit sequence to incorporate new glycosylation sites also favored formation of inhibin, collectively suggesting that overall glycosylation levels also regulate inhibin versus activin formation [43]. Activin itself can stimulate both α and β subunit synthesis suggesting that further internal feedback amplification once activin is produced [44]. In addition, BMP4 and 7 increase activin A expression and production in human granulosa cells [45]. Thus, regulation of inhibin/activin subunit synthesis is both complex and multifactorial, which accounts for the complicated patterns of these hormones in both animal and human studies.

While inhibin is clearly an endocrine hormone with primary actions regulating FSH biosynthesis and release from the pituitary, activin is more likely to be an autocrine/paracrine acting hormone [31]. Within the ovary, activin has been ascribed a number of activities including regulation of germ cell development, primordial follicle assembly and activation (see below), regulation of follicle growth and maturation, promotion of granulosa cell proliferation, suppression of thecal androgen production, and promotion of FSH receptor expression and response [46]. Activin upregulates cyp26b1, which is a retinoic degrading enzyme, a process that regulates sperm mitosis in males [47]. Activin also regulates estrogen receptor expression [48]. In human granulosa-lutein cells from in vitro fertilization (IVF) hyperstimulation, activin promoted expression of P450 aromatase, FSH receptor, and

estradiol production while inhibiting StAR, LH receptor, and progesterone levels via Smad2 and 3 phosphorylation [49,50].

The autocrine/paracrine roles of activins within the ovary were more fully delineated using inducible Cre-Lox technology to selectively eliminate activin A or activin B in the adult. This was necessary since embryonic deletion of the βA gene resulted in pups that died within 24 h of birth due to numerous developmental defects [51]. Although germ-line βB deficient mice survived to adulthood, offspring died perinatally perhaps due to insufficient lactation [52]. Granulosa cell-specific deletion of the βA gene resulted in subfertility due to enhanced corpus luteum survival that reduced and eventually eliminated estrous cycling [53]. When these mice were crossed with germ-line βB deletion mice, the resulting double knockout females were completely infertile and also accumulated large number of corpus lutea, suggesting that there is substantial redundancy between the βA and βB genes in terms of ovarian function [53]. Using the same strategy, conditional knockout of Smads 2 and 3 in ovarian granulosa cells individually had little detectable effect on ovarian physiology and overall fertility while the combined Smad2/3 knockout dramatically reduced fertility via disrupted follicular development, ovulation, and cumulus cell expansion of ovulated oocytes suggesting that these two second messengers have redundant functions in mediating activin signaling in the ovary [54]. Finally, knocking out the Smad4 common Smad that is required for complexing with phosphorylated Smads to transduce an activin signal produced a similar phenotype of subfertility and multiple defects in folliculogenesis including altered steroidogenesis [55]. These ovary-specific knockout models provide important clues to the roles of activins within the ovary to regulate follicle formation, development, and maturation.

Regulation of follistatin biosynthesis is incompletely understood and may differ depending on the tissue source, but for the most part activin, acting via Smad2 or 3 and FoxL2 appears to be a major simulator of follistatin biosynthesis in pituitary [56–58]. Pathways regulating follistatin synthesis in the ovary include protein kinases A and C [59], BMP2, and FoxL2 [60] as well as wnt/b-catenin [57,61] and Nrf2 [62]. Importantly, follistatin is synthesized as two mRNA transcripts, which produce three distinct follistatin proteins comprising 288, 303, and 315 amino acids in the mature protein [34]. Moreover, the FST288 protein binds tightly to heparin sulfate and thus, remains closely associated with cell surfaces within tissues while the FST315 form does not and is found mainly in the circulation [27,63]. The FST303 protein is mostly found in ovarian follicular fluid [34] and appears to be the dominant form acting in the ovary [18]. A recent study identified the liver as the source for circulating follistatin in humans [64].

The physiological functions of follistatin in the ovary are difficult to dissect given this complexity in biochemistry and the wide distribution of its synthesis within the body. Moreover, germ-line knockout of the follistatin gene resulted in neonatal lethality precluding identification of possible roles. Using the conditional knockout approach, however, follistatin was deleted from granulosa cells in adult ovaries which produced fertility defects including reduced litter number and size leading ultimately to infertility [65]. There was an overall reduction in follicle number along with elevated levels of serum FSH and LH. This study suggested that follistatin plays a key role in regulating follicle development and number and that when compromised, follicles eventually fail to develop and ovulate. Moreover, follistatin dysfunction might contribute to human infertility conditions, such as premature ovarian failure/primary ovarian insufficiency given the similarity of this condition to the phenotype of the granulosa cell-specific follistatin KO.

Studies in which the follistatin gene has been inactivated [65,66] do not address the relative roles of the different follistatin isoforms. To determine whether follistatin isoforms have distinct biological actions their properties were assessed individually [27]. Follistatin 288 (FST288) was found to be superior at regulating activin when expressed endogenously and bound to the cell surface compared to FST315 that did not bind to the cell surface [27]. This was true for activin whether applied exogenously or expressed from a transgene endogenously [27]. These observations suggested that FST288 may be sufficient for normal embryonic development given that animals with deletion of all follistatin isoforms were nonviable [66]. In addition, since the FST303 isoform is derived via C-terminal proteolytic processing of the full-length FST315 isoform, elimination of the alternative FST315 mRNA would lead to simultaneous loss of the FST303 ovarian isoform [34] and the circulating FST315 isoform [63] since it is derived by proteolytic processing of the FST315 tail [34].

To test this hypothesis, FST288-only mice were created by removing the alternative splicing sequences that create FST315 transcripts [67]. These mice were born in normal Mendelian ratios verifying that FST288 alone was indeed sufficient for development to adulthood in mice. Interestingly, these mice developed a fertility defect characterized by reduced litter size and frequency, reduced numbers of follicles, and early termination of fertility [67], similar to the granulosa-specific follistatin knockout [65] and to human premature ovarian failure/primary ovarian insufficiency. Detailed follicle counting demonstrated a larger number of primordial, primary and secondary follicles at 8.5 days and a reduced number of healthy antral follicles at 100 and 250 days in FST-288-only females demonstrating a more rapid demise of remaining primordial/primary follicles that accounted

for the early cessation of fertility [67]. In a follow-up study, these females demonstrated that the process of germ cell nest breakdown was extended in duration while apoptosis of forming antral follicles was reduced so that these females were born with more germ cells within the nests [68]. Taken together these studies demonstrate that follistatin is critical for regulating the process of follicle formation and activation, alteration of which leads to reduced fertility and early loss of reproduction. The critical importance of activin signaling in regulating this process of germ cell formation, nest breakdown, follicle formation, and follicle activation/maturation has been amply demonstrated in both mouse and human ovaries [69–71]. Taken together, these studies collectively demonstrate that one critical role of follistatin in the ovary is to regulate activin-mediated germ cell formation, development, and maturation.

HUMAN PHYSIOLOGY AND CLINICAL IMPLICATIONS

FSH Regulation for Controlled Follicle Development

Changes in inhibin secretion are critical for the precise regulation of FSH and development of a single dominant follicle in each menstrual cycle. FSH levels begin to decline approximately 1 day before peak inhibin B levels in the follicular phase. A time-lag analysis demonstrates an inverse relationship between FSH and inhibin B levels, suggesting that inhibin B may be the most proximate regulator of FSH at this time in the cycle [72].

Evidence for the negative feedback role of inhibin B also comes from the selective FSH rise that occurs in the early follicular phase during normal reproductive aging. The rise in follicular-phase FSH levels is associated with decreased inhibin B levels that result from the decreased small antral follicle number that occurs in ovarian aging [72,73]. The decrease in inhibin B levels occurs earlier than changes in estradiol and inhibin A, the other granulosa cell factors that control FSH, suggesting that the decrease in inhibin B is responsible for the FSH increase during aging [73–76]. These data provide indirect evidence for a role of inhibin B in FSH restraint.

In the late luteal phase, the decline in inhibin A levels coincident with the rise in FSH levels across the luteal follicular transition also suggest a role for inhibin A in FSH negative feedback. Inhibin A infusions in the luteal phase suppressed FSH and prevented the FSH rise at menses in the nonhuman primate, providing direct evidence that inhibin A is an important feedback regulator [77]. Similarly, daily inhibin A injections in the follicular phase in the nonhuman primate suppressed bioactive FSH levels [78]. On the contrary, maintenance of a midluteal phase estradiol level in women prevented the FSH rise at the luteal-follicular transition despite a decrease in inhibin A levels [79], data have continued to cloud the importance of inhibin A as a negative regulator of FSH in women. Taken together, the evidence suggests inhibin B, and possibly inhibin A, contribute to the negative feedback which regulate FSH levels in the follicular phase of the menstrual cycle.

Ovarian Aging, Perimenopause and Menopause

Study of the menopause transition provides support for the role of inhibin B as a marker of ovarian aging and time to menopause in addition to evidence supporting its role as a negative regulator of FSH levels. The stages of reproductive aging have been categorized from early reproductive age through late post menopause based on menstrual cycle regularity, symptoms and hormone levels [80]. These easily identifiable stages allow prediction of time to menopause [80]. The stages are called the STRAW stages based on the Stages of Reproductive Aging Workshop at which they were defined [80]. Several studies demonstrated that inhibin B levels decrease across the STRAW stages of reproductive aging [81]. Therefore, inhibin B levels, along with AMH and antral follicle count, have been included in the STRAW staging system for the prediction of menopause [80].

In addition to its role as a marker of ovarian function across the menopause transition, inhibin may have effects on bone turnover in the perimenopause. Mouse studies and human cell line studies support a direct role for inhibin in suppressing osteoblastogenesis and osteoclastogenesis [82,83]. The effect occurs through blockade of activin and by blocking the stimulatory effects of bone morphogenic proteins (BMPs) [82]. These local effects are consistent with serum inhibin levels, which correlate inversely with markers of bone turnover and formation. For example, decreased inhibin levels are consistent with the increased bone turnover that occurs in the perimenopause [83].

Primary Ovarian Insufficiency

Given the known inhibin B decrease in the menopause transition, inhibin B has been evaluated as a marker for primary ovarian insufficiency (POI), previously termed premature ovarian failure. The condition is caused by premature loss of oocytes and follicles, resulting in low estradiol levels, amenorrhea and elevated FSH levels. One of the more common causes of POI results from an expansion in a CGG repeat in the 5′ untranslated region of the *FMR1* gene, termed a premutation. Inhibin B levels are lower in women who carry the premutation than in control women of the same age even before menstrual

cycles become irregular, suggesting that they have early ovarian aging [84]. A decrease in inhibin B is clearly seen in women with primary ovarian insufficiency caused by all etiologies, but its ability to identify women with impending POI is not greater than that of AMH [85]. Indeed, AMH appears to be a more sensitive marker of imminent ovarian failure [85].

However, inhibin B levels are particularly important in ovarian insufficiency caused by autoimmunity. Adrenal insufficiency and/or the presence of adrenal antibodies are demonstrated in a majority of women with proven autoimmune oophoritis because the autoimmune reaction targets enzymes common to adrenal and ovarian steroidogenic cells [86]. In early stages of primary ovarian insufficiency caused by autoimmunity, a specific lymphocytic infiltrate invades the steroidogenic theca cells of the follicle, with relative sparing of the granulosa cells [86,87]. In these early stages of autoimmune oophoritis, estradiol production is compromised by the lack of androstenedione and testosterone precursor from theca cells [88]. The elevated FSH levels, in the absence of estradiol negative feedback, continue to drive granulosa cell division and follicle growth with a rise in inhibin B [88]. Thus, high inhibin B levels in the presence of very low estradiol levels serve as an excellent marker of autoimmune oophoritis in the early stages of development [88,89]. Eventually, the lymphocytic infiltrate invades the structures surrounding the theca cells, causing greater follicle destruction and primary ovarian insufficiency.

Puberty

In human female neonates, there is a self-limited rise in gonadotropins that stimulates increased inhibin A and B levels for approximately 6 months [90]. Inhibin A levels then decrease to undetectable levels at 6 months after birth, whereas inhibin B levels decrease to detectable but low prepubertal levels. Inhibin B levels are slightly higher from age 6 to 10 years but it is not until approximately age 10 years, in the years leading up to puberty, that inhibin B levels begin a more profound rise [91]. Anchoring inhibin levels to breast development in girls aged 6–11 years demonstrates that inhibin B levels increase gradually from Tanner stage I to II breast development, then increase more sharply to peak at Tanner stage III, indicating the maximum increase in follicle activity across reproductive age [92,93]. The peak inhibin B levels then fall slightly and plateau at stages IV and V as ovulatory menstrual cycles are established [92,93]. Inhibin B levels are correlated with those of FSH and LH levels from Tanner stage 1 through III breast development [93]. In contrast to inhibin B, inhibin A levels increase most dramatically at Tanner stage IV and are inversely correlated with FSH levels, indicating the onset of ovulatory cycles with corpus luteum activity. Taken together, the prepubertal and pubertal patterns of increase in inhibin B and A mark gonadotropin-dependent follicle development.

Based on the ability to mark follicle activity in puberty, inhibin B has been investigated as a biomarker of abnormal pubertal development. In women with delayed puberty from isolated hypogonadotropic hypogonadism, inhibin B levels were significantly lower than in control subjects in the early follicular phase, consistent with a smaller number of antral follicles. Inhibin B also has excellent sensitivity and specificity to distinguish hypogonadotropic hypogonadism from constitutional delay of growth as a cause of delayed puberty [94]. Inhibin B may serve as a useful indicator of pubertal progression and potential fertility in girls with Prader Willi syndrome, with utility as a clinical marker of the need for contraception [95]. Finally, inhibin B has been evaluated as a marker for precocious puberty although it was unable to differentiate premature thelarche and precocious puberty [96].

Pregnancy and Labor

Inhibin A, activin A, and follistatin rise across pregnancy to reach maximum levels at 36 weeks [97]. All are expressed in the placenta. Inhibin A is also produced from the corpus luteum, which is maintained by hCG stimulation from the trophoblast. Activin A rises at the end of pregnancy as a product of the trophoblast and positively correlates with the onset of labor [98].

For unclear reasons, inhibin A is elevated in pregnancies with Down syndrome and has been used for second trimester Down syndrome screening. A recent Cochrane analysis suggests that the small number of studies that used second trimester total hCG, unconjugated estriol (uE3), alpha fetoprotein (AFP), and inhibin A in combination had greater diagnostic accuracy compared to other test combinations that involved only one serum marker or nuchal translucency on ultrasound in the first trimester [99].

Inhibin A and activin A have been evaluated as early second trimester markers of preeclampsia. However, neither has the sensitivity nor specificity that is necessary for use in clinical practice [100]. Inhibin A and activin A have also been evaluated in multimarker panels to identify the location of a pregnancy, whether intrauterine or ectopic, with activin A more promising as a marker of ectopic pregnancy [101]. However, none of the marker panels are yet accurate enough for clinical use.

In Vitro Fertilization and Ovarian Follicle Reserve

Evidence that inhibin B derives from the developing cohort of follicles in the menstrual cycle prompted its

evaluation as a prognostic marker of follicle development during assisted reproductive technologies. Basal prestimulation and early stimulation levels that predict subsequent ovarian responsiveness and pregnancy provide the most useful predictive information. Initial studies using basal inhibin B levels were promising and the inhibin B response to an exogenous FSH ovarian reserve test was predictive of ovarian reserve [102]. However, the majority of studies do not find baseline or stimulated inhibin B levels useful as a screening test for assisted reproductive technologies [103,104]. There is a great deal of overlap in inhibin B levels in subjects who have a good response to treatment compared to those who do not [105]. Taken together, baseline inhibin B and inhibin B during stimulated screening tests do not match the clinical utility of the more easily measured AMH levels.

Ovarian markers are also needed as predictors of ovarian function after chemotherapy. Recovery of ovarian function depends on age, the type of chemotherapy, and the pretreatment ovarian reserve. In some studies, inhibin B in combination with AMH predicts ovarian function after chemotherapy for breast cancer [106,107]. Further evaluation of its use as a predictor is necessary.

Ovarian Cancer

The majority of ovarian cancers, approximately 90%, derive from ovarian surface epithelium, while 5%–10% arises from granulosa cells. As the inhibins are produced by granulosa cells, the secretion of inhibins is greatest from granulosa cell tumors and inhibin serves as an important marker [108]. Serum inhibin levels are also elevated in mucinous ovarian tumors [108]. A study comparing inhibin levels using a variety of inhibin assays demonstrated that inhibin assays directed to the α subunit of inhibin, which measures both free α subunit and dimeric inhibin, detected 100% of granulosa cell tumors and 70%–90% of mucinous epithelial ovarian carcinomas [108]. The dimeric inhibin B assay also detected over 90% of granulosa cell tumors, but was less useful for detecting mucinous epithelial ovarian tumors [108]. Finally, the utility of the α inhibin subunit directed assay and dimeric inhibin in monitoring granulosa cell tumor recurrence has been established for both juvenile and adult granulosa cell tumors [109,110].

In other ovarian cancers derived from the ovarian surface epithelium, inhibin is less commonly secreted. Total inhibin α subunit directed assays are better than the other inhibin assays at detecting the serous epithelial ovarian tumor subtypes and miscellaneous ovarian tumors although CA125 is superior to inhibin in these cases [108]. A combination of CA125 and an α inhibin subunit directed assay increases the sensitivity of detection slightly but has not been used clinically [108].

In addition to serving as a marker for malignant sex-cord stromal tumors, dimeric inhibin secretion can cause unusual menstrual cycle disruptions in cases of benign sex-cord stromal tumors. Dimeric inhibin secretion has been demonstrated from benign sex cord stromal tumors, such as fibrothecomas [111]. These tumors may result in irregular menses or amenorrhea in a premenopausal woman or lower than expected FSH levels in a postmenopausal woman as a consequence of the FSH suppression by dimeric inhibin. Tumor removal can restore menstrual regularity in these rare cases in reproductive age women.

Polycystic Ovary Syndrome

PCOS is a disorder defined by two out of three criteria: irregular menses, hyperandrogenism, and polycystic ovary morphology on ultrasound. Follicles are arrested at a small antral stage of development. Although the number of follicles in polycystic ovaries is increased compared to normal ovaries, the majority of studies examining serum inhibin levels in women with PCOS have failed to demonstrate increased inhibin A or inhibin B levels [112,113]. These findings suggest that inhibin B is relatively decreased per follicle in women with PCOS. Consistent with the hypothesis, follicular fluid inhibin A and inhibin B levels are lower in women with PCOS compared to control women [114].

When hormonal and metabolic factors were examined in relation to inhibin levels in women with PCOS, inhibin B levels, but not inhibin A levels, were inversely correlated with BMI and factors related to BMI including LH, insulin, and SHBG [112,113]. As expected based on the correlations, short-term insulin suppression with diazoxide increased inhibin B and hCG administration suppressed inhibin B secretion [112,115]. Recent genome-wide association studies identified a gene variant near the *FSHβ* gene that is associated with PCOS, lower FSH and higher LH levels [116]. Thus, the slightly lower FSH or higher LH levels may explain the lower inhibin B levels in PCOS.

Activin and follistatin levels also demonstrate abnormalities in PCOS. In general, follistatin levels are increased in women with PCOS [117,118]. Activin, in contrast, has been reported as decreased or not different [118]. There is no direct evidence that activin plays a role in the pathophysiology of PCOS. However, the possibility that follistatin plays a role in the pathophysiology is still under investigation.

SUMMARY

Now that the genome has been sequenced we know that no additional inhibin/activin/follistatin genes will be discovered. Therefore, future progress will most likely

come from a better understanding of the physiological roles of each of these proteins both in local, autocrine/paracrine actions and in endocrine activities. For example, are the different molecular weight forms of dimeric inhibins equal in activity at the pituitary? Do they have different roles within the ovary? Is there any endocrine role for serum activin and follistatin or does the complex in serum merely represent hormones on their way to disposal? Is there a physiological mechanism that can release activin from follistatin since natural dissociation of the complex is essentially nonexistent? How exactly does inhibin block activin signaling? How does the relatively small concentration of inhibin in serum regulate activin action at the pituitary when it binds to the activin receptor with affinities more than 10-fold lower than would be expected for the known inhibin concentration? Are there accessory proteins that have not yet been identified to account for this discrepancy?

Clinically, inhibin A serves as a standard marker for Down syndrome in the second trimester of pregnancy. Inhibin α subunit has excellent utility as a biomarker of granulosa cell and mucinous ovarian tumors. Inhibin B levels mark active autoimmune oophoritis at its early stages, before the onset of primary ovarian insufficiency. In contrast, inhibin B levels seemed promising as a fertility marker and as a marker of polycystic ovary morphology. However, AMH levels are superior for assessment of the follicle complement. Prospective trials will determine the utility of inhibin A and activin A for the diagnosis of ectopic pregnancy, inhibin B for the diagnosis of hypogonadotropic hypogonadism and inhibin α subunit as a marker of mucinous ovarian tumor recurrence.

Thus, the inhibin field has matured substantially. However, there is still a long way to go to fully understand ovarian physiology and clinical utility of the inhibin family of proteins.

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