MINIREVIEW

Follistatin and Activin: A Potential Intrinsic Regulatory System within Diverse Tissues

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Until the 1920s, it was assumed that the sole gonadal hormones modulating anterior pituitary glandular secretions were lipid-soluble entities called steroids. Experimental evidence then surfaced during the ensuing decade that demonstrated that the testis also contained a hydrophilic material that might also influence pituitary secretory activity (1, 2). The name "inhibin" was given to this novel substance in view of its ability to inhibit hypertrophy of pituitary cells in castrated rats (2). Work in later years evoked the concept that inhibin could be an important regulator of follicle-stimulating hormone (FSH) secretion (3, 4). Thus, as a consequence of these landmark studies, investigators began a search to elucidate the chemical nature of the inhibins.

Although inhibin was discovered as a potential secretory product of the testis, it was almost 45 years later that a nonsteroidal FSH suppressor was demonstrated in the ovarian follicular fluid of cows (5). Ironically, it was the ovarian follicular fluid from which chemists eventually isolated what was most likely the putative inhibin molecule described in the 1920s and 30s (6–9). Chemical characterization of the purified FSH-suppressing material revealed that inhibin is a 31- to 32-kDa heterodimer consisting of two distinct polypeptide chains, α and β. In pig follicular fluid, two inhibin heterodimers, A and B, were isolated and found to consist of a common α-subunit chain linked by disulphide bridges to a β_A- or β_B-subunit chain, respectively. Both inhibins were essentially equipotent in suppressing FSH secretion in the dispersed anterior pituitary cell bioassay system (6).

At long last, the elusive inhibin had been proven a true chemical entity—a major breakthrough in reproductive endocrinology. However, little did anyone know that the gonads produced other FSH-regulatory polypeptide factors besides inhibin, and that the ability of these other factors to modulate FSH secretion could be only the tip of the iceberg with regard to the multifunctional properties of these factors, thereby extending their importance to other areas of biology. It is these other factors and their potentially important relationship to each other that is the primary focus of this review. Although the biological actions of inhibin will be alluded to, several excellent review articles primarily devoted to the chemistry and biology of inhibin have been published in recent years (10–12).

Discovery of Follistatin and Activin

Two years after the chemical identification of inhibin, it was discovered that the FSH-suppressing activity of ovarian follicular fluid may not be due entirely to the inhibins. Two laboratories independently uncovered another compound that could inhibit FSH secretion, although it was less potent than the inhibins and was structurally unique (13, 14). This new compound was given the name FSH-suppressing protein (14), or, as it is more commonly referred to, follistatin (13). In contrast to inhibin, follistatin is a single-chain, glycosylated protein present in follicular fluid in at least three isoforms of 31–32, 35 and 39 kDa (13, 14), all with equal potency in inhibiting FSH secretion.

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In addition to the two FSH-suppressing materials, follicular fluid also harbored factors that were capable of stimulating FSH release. Two laboratories identified the chemical structure of these FSH stimulators and appropriately named them activins (15, 16). Interestingly, the activins were dimers of the β-subunits of inhibin. The homodimer of the β_A-chain was designated activin-A, and the $\beta_A\beta_B$-heterodimer was designated activin-AB. Subsequent to the identification of the ovarian activins, an erythroid differentiation factor was also isolated from conditioned medium of the THP-1 human monocytic cell line and found to be identical to activin-A (17, 18). In addition, the mouse (19) and frog (20) homologs of activin-A have recently been isolated from WEHI-3 murine myelomonocytic leukemia cells and *Xenopus* XTC cells, respectively, and found to be potent mesoderm-inducing factors. Moreover, a separate induction factor for body axis patterning present in frog (21) and chicken (22) blastulae may be a homolog of the mammalian activin-B homodimer ($\beta_B\beta_B$), since activin mimics the inductive effects of this factor, and expression of the inhibin-β_B subunit gene in inductive hypoblast of the chick embryo commences at the time axial patterning is initiated (22).

Table I. Distribution of Inhibin β-Subunit and Follistatin mRNA in Rat Tissues[a]

Group[b]	Tissue	β_A-Subunit	β_B-Subunit	Follistatin
1	Ovary	+	+	+
	Testis	+	+	+
	Pituitary	−	+	+
	Adrenal	+	−	+
	Brain	+	+	+[c]
	Bone Marrow	+	−	+[d]
2	Spleen	+	−	−
	Placenta	+	+	?
3	Heart	?	?	+
	Lung	?	?	+
	Thymus	?	?	+
	Skeletal Muscle	?	?	+
	Gut	?	?	+
	Uterus/decidua	?	?	+
	Kidney	−	−	+
	Pancreas	−	−	+
4	Liver	−	−	−

[a] Information compiled from references 61 through 64. +, mRNA present; −, mRNA not detectable; ?, tissue not analyzed for mRNA.
[b] Group 1, both β-subunit and follistatin mRNA; Group 2, β-subunit mRNA only; Group 3, follistatin mRNA only; Group 4, neither β-subunit nor follistatin mRNA detected.
[c] Cortex, cerebellum.
[d] See Figure 1 of this review.

Multifunctional Properties and Localization of Activin and Follistatin

In addition to stimulating FSH synthesis and release, activin has a broad range of activities that includes effects on cell growth, differentiation, and maturation. These varied actions on the growth and differentiation of a variety of cell types no doubt reflect the partial sequence homology of the inhibin-β subunits to an ever-growing family of growth/differentiation factors that includes transforming growth factor-β (23), the product of the decapentaplegic gene complex in drosophila (24), müllerian-inhibiting substance (25), the *Xenopus* protein Vg-1 (26), and bone morphogenic proteins 2 through 6 (27). A list of specific activities thus far ascribed to the activins, along with the appropriate reference numbers, is presented in Table I. While viewing this table, the reader should keep in mind that many of these actions require confirmation by other laboratories. A case in point is the failure of Tsafriri *et al.* (60) to observe an effect of activin-A on the spontaneous maturation of oocytes, even though Itoh *et al.* (52) subsequently reported stimulation of oocyte maturation by activin-A.

In view of the wide spectrum of activities exerted by the activins, it is not too surprising that the mRNA encoding the inhibin-β subunits is found in a multitude of diverse tissues, including the gonads, pituitary, placenta, and bone marrow (Table II). However, while data on individual subunits often lead one to conclude that activin dimer is produced in a particular tissue, additional data are necessary before such claims can be considered scientifically accurate. Tantamount to such claims are data which demonstrate that the gene products are actually transcribed and that the dimer actually exists in the tissue. Until now, β-subunit protein has been visualized by immunohistochemistry only in the brain (65), gonads (66–70), and pituitary gland (71). On the other hand, activin dimers have yet to be demonstrated in a normal mammalian tissue or fluid, other than ovarian follicular fluid from which it was originally purified and the conditioned media of several cell lines (see above). With the exception of bone marrow, in which only the β-subunit gene is expressed, the demonstration of activin dimer in tissues is critical, since the tissues may produce only $\alpha\beta$ dimers (66). Should activin dimers eventually be demonstrated in tissues expressing β-subunit genes, then the fact that tissues that produce activin (i.e., gonads, pituitary, and marrow) are also major target tissues for activin's actions leads one to propose that activin is primarily a modulator at the local level. Nonetheless, if the recent explosion of information regarding the multiple actions of activin is any indication, then one can safely predict that new functions and tissue distributions for activin and its subunits will be discovered in the very near future, such that both Tables I and II will require updating by the time this minireview sees the printed page.

In sharp contrast to the activins, there have been only a few actions of follistatin that have been docu-

Table II. Multifunctional Properties of Activin in Mammals

Effect	Tissue	Action	References
Proliferation	P19 embryonal teratoma cells		28, 29
	Human luteinized granulosa cells		30
	Fetal rat osteoblasts		31
	Rat spermatogonia		32
Antiproliferation	K-562 human erythroleukemic cell line		19, 20, 33, 34
	Erythroid progenitor cells in normal human bone marrow		33
	Mice BALB/c 3T3 cells		35
	Rat thymocytes		36
	Chinese hamster ovary (CHO-K1) cells		37
	Rat pituitary somatotropes in response to growth hormone-releasing hormone		38
	Human fetal adrenal cells		39
Differentiation	K-562 human erythroleukemic cell line	Hemoglobin accumulation	18, 33, 34
	L8057 murine megakaryoblastic cell line	Induction of acetylcholinesterase	40
	Hematopoietic progenitor cells of human bone marrow	Enhance colony formation	41, 42
	Erythroid progenitor cells of bone marrow	Stimulate erythropoiesis:[a]	
		in rats	43
		in mice	44
	Rat ovarian granulosa cells	Augmentation of FSH-stimulated estrogen and progesterone production	45, 46
		Augmentation of FSH-stimulated LH receptor induction	45
		Stimulation of FSH receptor induction	47
		Stimulation of inhibin secretion	45, 48
		Stimulation of inhibin subunit mRNA	48
	Bovine ovarian granulosa cells	Inhibition of oxytocin and progesterone secretion	49
	Rat testicular Leydig cells	Inhibition of LH/human chorionic gonadotropin-stimulated testosterone production	50, 51
Maturation	Rat oocyte	Stimulate meiotic maturation	52
Hormonogenesis	Rat anterior pituitary cells	Inhibit basal and growth hormone-releasing hormone-stimulated GH secretion	38, 53, 54
		Inhibit thyrotropin-releasing hormone-stimulated prolactin release	54
	Rat pancreatic islets	Stimulate insulin secretion	55
	Human placental cells	Increase release of LHRH and progesterone	56
Other	Rat hepatocytes	Stimulate glucose production	57
	Rat ovarian follicles	Atresia[a]	58
	Human monocytes	Increase migration activity	59

[a] *In vivo* effects.

mented, other than its FSH-suppressing action. These actions include diminution of FSH-stimulated estrogen and inhibin secretion from cultured rat ovarian granulosa cells (46), augmentation of FSH-stimulated progesterone secretion from cultured granulosa cells (46), and a modest suppression of pituitary luteinizing hormone-releasing hormone (LHRH) receptors (72). Despite the paucity of actions thus far ascribed to follistatin, follistatin mRNA transcripts have been detected in no less than 14 different tissues, including our recent detection of follistatin mRNA in bone marrow using the polymerase chain reaction (Fig. 1 and Table II). Indeed, this diverse distribution could predict that follistatin, like activin, has tissue-specific effects or that follistatin may have a universal action on different cell types. Follistatin protein, on the other hand, has been demonstrated only in ovarian tissue (73, 74; see below) and anterior pituitary gland (75, 76). Interestingly, little or no follistatin was detected by immunohistochemistry in the kidney using a polyclonal antibody raised in our laboratory, in spite of an abundance of follistatin message in this organ (62, 64; authors' unpublished observations). Clearly, further work is necessary and is currently underway to determine whether follistatin protein is present in tissues expressing the follistatin gene.

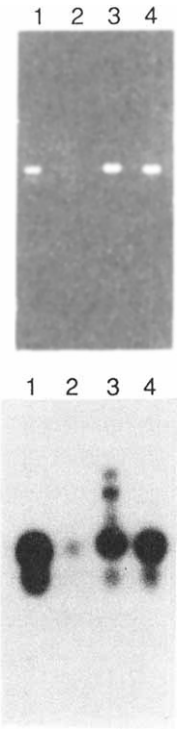

Figure 1. (Top) Amplification of follistatin mRNA target sequences in rat femur bone marrow by polymerase chain reaction (PCR). Total RNA was extracted from bone marrow of female rats and used to prepare single-stranded cDNA. The cDNA was used as template for PCR using the two primers 5′-GAG TGT GCC ATG AAG GAA G′C-3′ and 3′-CTT CTG GTC CTG ATG TCG AA-5′, encoding follistatin amino acid sequences Glu^{265}-Cys-Ala-Met-Lys-Glu-Ala^{271} and Glu^{301}-Asp-Gln-Asp-Tyr-Ser-Phe^{307}, respectively, to obtain the targeted 127-bp fragment. PCR products (100 ng) were examined by gel electrophoresis using a mixture of 3% NuSieve GTG agarose and 1% Sea Kem ME agarose (FMC Bioproducts, Rockland, ME). Lane 1 corresponds to the positive control from a rat follistatin cDNA clone (62), Lane 2 is the negative control from a DNA-free sample, and Lanes 3 and 4 correspond to cDNA prepared from intact and hypophysectomized female rat marrow, respectively. (Bottom) Southern blot analysis of PCR products from the upper panel, with the exception of Lane 2, in which 10 ng of PCR product from intact marrow cDNA were electrophoresed.

Relationship of Activin to Follistatin

By the time follistatin was isolated and characterized, it was clear that inhibin was a major endocrine regulator of FSH secretion, at least in the rat. What then was the purpose of a separate FSH-suppressing protein that was less potent than inhibin? Surely it did not make much sense for the ovary (or testis) to release the less potent follistatin when it could release fewer inhibin molecules into the circulation to effectively regulate FSH biosynthesis and release. Indeed, follistatin was perceived by many as being just a weak inhibin agonist, a quirk of nature so to speak. In spite of this apparent apathy for follistatin and for lack of a better approach, we embarked on a series of studies with the goal of defining a classic endocrine role for follistatin in regulating FSH secretion. About the time these studies commenced, a report appeared which showed that bovine pituitary folliculostellate cells secreted follistatin (75), thus implying that the role of follistatin in regulating FSH secretion may be as a local, rather than as a classical, endocrine modulator. A subsequent study, however, would chart the course for future studies on follistatin. In search of activin receptors in the rat ovary, Nakamura *et al.* (73) instead uncovered a binding protein for activin that was structurally identical to follistatin. Thus, as a consequence of this landmark study, the biology of follistatin became intertwined with the biology of the activins.

Despite documentation of the many diversified actions of activins, we know little about the true physiological import of these intriguing dimers. Consequently, there is little to guide the investigator as to the potential physiological implications of follistatin as an activin-binding protein or vice versa. Nonetheless, we have decided to pursue our studies concerning the biological implications of the follistatin-activin relationship from the perspective of a local regulatory control system. This approach was chosen based on the following observations. First and perhaps foremost, in terms of our decided course of study, was the widespread tissue distribution of inhibin-β subunit and follistatin mRNA (Table II). As alluded to earlier, this implies, but does not conclusively demonstrate, that activin and follistatin proteins are made locally. Furthermore, the potential for local production of a particular factor by a given tissue does not in itself exclude the possibility that the same factor produced extrinsically (by another

tissue) may act as a classical endocrine modulator on that tissue. Such is likely the case for inhibin, in which the presence of α- and β-subunit proteins in the anterior pituitary gland suggests local production of the $\alpha\beta$ dimer, yet it has been established, at least in the rat, that inhibin of ovarian origin is an important endocrine modulator of FSH secretion (10).

This brings us to our second observation that neither follistatin nor activin appears to circulate in the bloodstream in amounts that would warrant their classification as endocrine modulators. This statement is based on our recent preliminary observations using a follistatin radioimmunoassay and indirect evidence concerning the FSH-suppressing activity of rat ovarian venous plasma. In the case of follistatin, serum follistatin levels in rats were found to be very low, approximating only 100 fmol/ml (value expressed in terms of a highly purified porcine follistatin standard [77]), in contrast to immunoreactive inhibin levels in adult, cycling female rats, which vary between 500 and 2000 fmol/ml (78, 79). Moreover, the serum level of follistatin is relatively the same irrespective of cycle stage, sex, and whether the gonads, anterior pituitary gland, or one kidney is removed from the rat. With regards to activin, when ovarian venous plasma collected from rats at various times during the estrous cycle was charcoal-extracted and added to a dispersed pituitary cell bioassay system, the plasma either significantly inhibited basal (non-LHRH stimulated) FSH secretion or had no effect (80–82). At no time did the plasma significantly stimulate FSH secretion. Since the bioactivity of the plasma likely reflects the balance of FSH-stimulating and inhibiting factors potentially being secreted by the ovary, one could infer from these data either that activin is not secreted by the ovary or that activin is secreted in amounts insufficient to offset the inhibitory effects of inhibin. The latter case, however, would appear inconsistent with a role for activin as an endocrine regulator of FSH secretion.

For the remainder of this review, we would like to present recent evidence that implicates the potential existence of a local regulatory system within two diverse tissues involving the interaction of activin and follistatin. The tissues that have been chosen are the anterior pituitary gland and the ovary. As can be seen from Table II, the genes encoding the primary structure of one or both inhibin-β subunits, as well as of follistatin, are expressed in these tissues. Although our subsequent discussions will focus on activin and follistatin, it is not our intention to disregard or downplay the importance of inhibin in regulating certain functional aspects of these tissues. Rather, we will attempt to integrate as best we can the evidence for local regulatory control of tissue-specific functions by activin and follistatin into the overall regulatory schema, which undoubtedly includes inhibin to one degree or another. When considered in this light, however, it will become increasingly evident to the reader that one of the major challenges in establishing the activin-follistatin relationship as an important local regulatory system is to distinguish the relative participation of local follistatin versus both local and systemic inhibin in the total regulatory scenario, since in addition to inhibiting FSH release, inhibin and follistatin may share other biological actions.

Anterior Pituitary Gland

Like all hormones of the anterior pituitary (AP), FSH synthesis and release are controlled to a significant extent by neuropeptides secreted into the pituitary portal vasculature from nerve terminals of the median eminence. The most important neuropeptide in this regard is LHRH, although the existence of a separate releasing factor for FSH has been proposed for several decades (83). However, there has been mounting evidence that indicates that a portion of FSH secretion is autonomous, i.e., independent of diencephalic influences. Such lines of evidence stem from both *in vivo* (84, 85) and *in vitro* (86) data showing a persistence of FSH secretion in the face of low to immeasurable secretion of luteinizing hormone (LH). The divergence of LH and FSH secretion is also observed during the natural estrous cycle of rodents. For example, preovulatory increases in LH and FSH secretion on proestrous afternoon are followed by a second increase in FSH secretion, but not in LH secretion, on estrous morning (78, 80). The primary stimulus for this second rise in circulating FSH levels has been shown to be the decreased secretion of ovarian inhibin that occurs during the afternoon and evening of proestrus, thereby releasing the FSH-containing gonadotropes from negative feedback inhibition (78–80). Whereas preovulatory increases in circulating gonadotropin concentrations may be abolished by ablation of the hypothalamus (87), removal of the diencephalon (88), or administration of potent LHRH antagonists (87, 89) or antisera (90, 91) prior to the initiation of the preovulatory surges, such experimental manipulations, when performed after the preovulatory LH and FSH surges but prior to the second FSH increase, fail to influence this portion of periovulatory FSH release. Coupled with the potent direct inhibitory effects of inhibin on AP FSH synthesis and secretion, these data strongly imply that while centrally mediated preovulatory LH and FSH surges are essential for the fall in ovarian inhibin secretion and consequent elevation in FSH levels on estrus, this second phase of FSH secretion, which is important for recruiting new follicles for ovulation in the next cycle, is an event intrinsic to the AP.

Another experimental condition in which circulating FSH levels are elevated independently of LH is shortly after ovariectomy (OVX) of rats on diestrus-1 of the cycle. Following removal of the ovaries, increased

levels of circulating FSH are observed by 4 hr whereas initial increments in LH levels generally occur 1–3 days later (92, 93). Administration of an LHRH antagonist 24 hr before OVX revealed that a significant component of the acute FSH hypersecretory response to OVX was readily apparent, despite neutralization of LHRH bioactivity (93). This suggested to us that the LHRH-independent secretion of FSH might represent an autonomous component of the total FSH regulatory system, mediated perhaps by a factor(s) produced and secreted by pituitary cells themselves acting in a paracrine and/or autocrine fashion.

To test this hypothesis further, it was considered desirable to use an animal model in which the AP is isolated from direct central nervous system intervention such that AP responses to OVX may be studied without the confounding influences of endogenous neural secretions. An animal model that meets this criteria is the hypophysectomized rat bearing AP allografts underneath the kidney capsule. To ascertain whether OVX can elicit FSH hypersecretion from AP allografts, hypophysectomized/grafted (H/G) rats, as well as H rats not bearing AP grafts, were injected with pregnant mare's serum gonadotropins (PMSG) 4–7 days following transplantation of AP tissue. Control H and H/G rats received the saline vehicle. The PMSG was given to some animals in order to enhance the secretion of negative feedback effector substances, such as estradiol-17β and inhibin, since serum levels of these hormones in H or H/G rats are extremely low due to the lack of gonadotropic stimulation of the ovaries (94, 95). Thus, it was reasoned that a response to removal of negative feedback signals, in this case the increase in FSH secretion, can only be observed if such signals are present initially. Forty-eight hours later, rats were either ovariectomized or sham castrated and blood was collected from indwelling atrial catheters inserted 1 day earlier. As shown in Figure 2, OVX of H rats bearing three AP allografts elicited an FSH, but not LH (data not shown), hypersecretory response, not unlike the divergent secretion of the gonadotropins observed acutely after OVX of pituitary-intact rats (92, 93). No such increases in FSH occurred in H/G rats given saline or in H rats given either PMSG or the saline vehicle (95). These data, when taken in the context of other evidence cited in preceding paragraphs, provide unequivocal support for the expression of an autonomous component of FSH secretion from pituitary-intact rats following OVX and during the secondary FSH increase, and further indicate that such central nervous system-independent secretion is activated by declining levels of inhibin (and, to a lesser degree, estradiol) in the circulation.

Having addressed the first part of our hypothesis, concerning whether autonomous FSH secretion can occur from AP tissue *in vivo*, what is the current evidence regarding the potential participation of locally derived activin in regulating autonomous secretion of FSH? First, inhibin β_B-subunit mRNA levels in the AP are elevated 3 weeks after OVX relative to pre-OVX levels (71). However, since α-subunit mRNA levels are also increased after OVX, one cannot establish based on this piece of evidence whether OVX increases the production of inhibin-B or activin-B or both. More convincing evidence for mediation of autonomous FSH secretion by activin is provided by the finding that basal FSH secretion from cultured AP cells is suppressed by co-incubation with a monoclonal antibody directed against activin-B (96). Although these latter data were derived from *in vitro* experiments, evidence from the H/G rat model would appear consistent with a role for activin in mediating autonomous FSH secretion. One day after OVX, H/G rats treated previously with PMSG were injected with 60 μg of purified porcine follistatin or saline. As depicted in Figure 3, serum FSH levels were suppressed to 63% of levels measured prior to injection, whereas levels of this gonadotropin were not altered by saline injection (95). Since a major portion

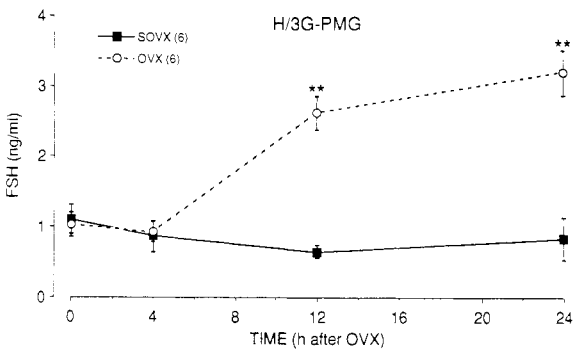

Figure 2. Pituitary FSH response to sham OVX or OVX in H rats bearing three pituitary allografts (3G) treated 48 hr previously with 30 IU PMSG (PMG). Numbers in parentheses represent the number of animals per group. Each point and vertical bar represents the mean ± SE. ** $P < 0.01$ vs sham control (from Ref. 95).

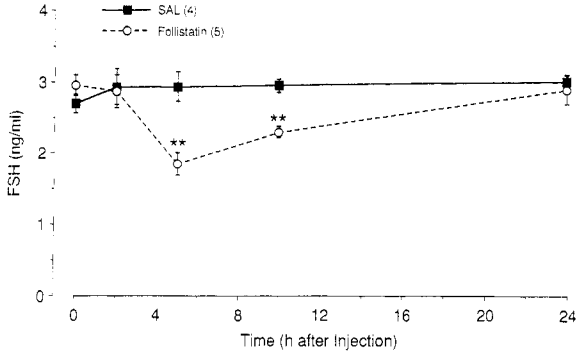

Figure 3. Effects of highly purified porcine follistatin on OVX-induced FSH hypersecretion in PMSG-treated H/G rats. Hypophysectomized rats bearing two pituitary transplants received 30 IU PMSG and were castrated 48 hr later. Twenty-four hours after OVX, rats received either Saline or 60 μg of follistatin iv. ** $P < 0.01$ vs saline-treated rats. See Figure 2 for further details (from Ref. 95).

of the FSH-suppressing action of follistatin is seemingly attributed to its ability to bind activin (96), these *in vivo* data provide substantive support for a role of activin in mediating enhanced autonomous FSH secretion. It should be noted that while the activin-B antiserum does recognize free subunit as well as dimer (Drs. T. Woodruff and J. Mather, personal communication), follistatin binds only to intact dimer (Fig. 4). Nonetheless, considering that inhibin α- and β_B-subunit immunoreactivity are localized exclusively within the gonadotropes (71), regulation of FSH by AP-derived activin would appear to be autocrine in nature.

Evidence for participation of locally produced follistatin in regulating FSH secretion is at best suggestive to this point. As alluded to earlier, follistatin has been identified as a secretory product of bovine folliculostellate cells (75) and follistatin mRNA transcripts have been demonstrated in the pituitary gland (64). Studies are currently underway to examine the regulation of follistatin gene expression in the AP. However, the production of follistatin by folliculostellate cells raises an important issue regarding the potential local actions of follistatin given the widespread distribution of folliculostellate cells in the AP (97). In addition to producing factors with paracrine actions on other AP cell types, additional functions have been ascribed to folliculostellate cells, including ones of support, growth, phagocytosis, and ion transport (97). Hence, it may be that follistatin influences an array of functions not only of the gonadotropes, but of other cell types as well, although it should be pointed out that other products of folliculostellate cells, such as basic fibroblast growth factor (98) and vasoactive intestinal peptide (99; Dr. A. Carrillo, personal communication), may mediate some of these diverse functions. Clearly, more comprehensive studies are required to address the potential significance of follistatin as a local modulator of FSH secretion in the AP, particularly in light of the well-established role of inhibin as an important endocrine modulator of FSH.

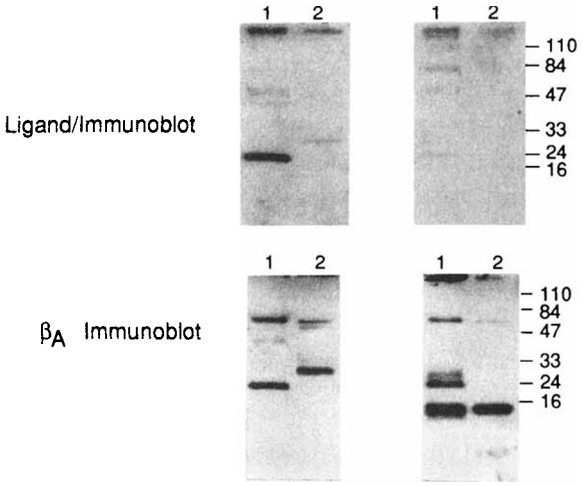

Figure 4. Specific recognition of activin-A homodimer by follistatin utilizing a ligand/immunoblotting procedure developed in our laboratory (top panels) and an inhibin-β_A subunit Western blot (bottom panels). Thirty nanograms of recombinant human activin-A and inhibin-A (supplied by Drs. T. Mason and R. Schwall, Genentech, Inc., South San Francisco, CA) were applied to Lanes 1 and 2, respectively, and electrophoresed on sodium dodecyl sulfate-polyacrylamide gel. The migration positions of prestained molecular weight standards run on an adjacent lane are indicated on the right ($M_r \times 10^{-3}$). Samples and standards were run in both blotting systems under nonreducing (left panels) and reducing (right panels) conditions. For the ligand/immunoblot procedure, the filter (after transfer) was first incubated with 5 n*M* purified porcine follistatin followed by incubation with a 1/500 dilution of an antiserum (Rb-32) raised in our laboratory against porcine follistatin. For the β-subunit immunoblot, the filter was incubated with a 1/500 dilution of an antiserum raised against a synthetic fragment (amino acids 90–107) of the inhibin-β_A subunit (provided by Dr. M. Culler, National Institute of Environmental Health Services, NIH). Following incubation with the antisera, the filters were washed and incubated with a second antibody conjugated to horseradish peroxidase and then treated with a mixture of luminol, *p*-phenylphenol, and hydrogen peroxide. Such treatment allowed chemiluminescent detection of the immunoreactive bands following development of the films. Note that the ability of follistatin to detect inhibin (top left) is far less than its ability to detect activin dimer. The differential binding of follistatin to activin and inhibin likely relates to our recent demonstration that activin has two binding sites for follistatin, whereas inhibin has only one binding site (117). In addition, note the absence of low molecular weight immunoreactive proteins in the ligand/immunoblot under reducing conditions (top right) in comparison to the detection of free β-subunit (M_r = 14,000) in the β-subunit Western blot (bottom right).

With regard to the regulation of other AP hormones, activin has been shown to inhibit basal and growth hormone-releasing hormone-stimulated growth hormone (GH) biosynthesis and secretion (Table I and 38, 53, 54). In marked contrast to its opposing actions on activin-stimulated FSH secretion, inhibin could not counteract the effects of activin on GH secretion (38). Interestingly, the suppressive effects of activin and somatostatin were additive, suggesting different mechanisms of action of activin and somatostatin to inhibit GH (53). Assuming that the production and secretion of activin are solely a property of the gonadotropes (71), the effects of activin on GH synthesis/secretion may indicate an important cell to cell communication link between gonadotropes and somatotropes, the physiological significance of which remains to be determined. However, if a communication link between these two cell types via activin does in fact exist, then one could theorize that activin-dependent increases in FSH secretion, as might occur in female rats during the secondary FSH surge or acutely after OVX, should be accompanied by decreases in GH secretion. A detailed examination of circulating FSH and GH levels during selective elevations of FSH may yield essential information that either supports or refutes this theory.

Ovary

As with the AP, much of the evidence purporting to the existence of a local regulatory system involving activin and follistatin within the female gonad is suggestive, but it is more substantive than the evidence for

the AP. Initial studies designed to examine possible local effects of activin and follistatin within the ovary were conducted *in vitro*, using granulosa cells obtained from immature rats given diethylstilbesterol for several days prior to sacrifice to induce the development of preantral follicles. These studies revealed that exogenous activin-A enhanced FSH-stimulated estrogen, progesterone, and inhibin production, as well as the induction of LH receptors by FSH (45, 46, 48). In addition, activin by itself stimulated induction of FSH receptors (47), inhibin-subunit gene expression, and secretion of immunoreactive inhibin (48). These effects of the inhibin-β dimers are likely mediated by specific receptors for activin (58, 100).

On the other hand, Xiao *et al.* (46) examined the effects of follistatin on granulosa cell differentiation and found that, in contrast to activin, follistatin suppressed FSH-induced increments in aromatase activity and inhibin secretion while augmenting FSH-stimulated progesterone production similar to activin. Since β-subunit and follistatin messages and proteins are co-localized in granulosa cells (see below), these *in vitro* data indicate that activin and follistatin may interact within the granulosa cell microenvironment to regulate certain differentiative processes. Interestingly, the effects of follistatin manifested on these ovarian cell types are similar to changes in cell function normally associated with cell demise (101, 102). An additional observation worth commenting on is the failure of inhibin to consistently alter granulosa cell steroidogenesis (45, 103, 104), thereby indicating that in contrast to the AP, in which the actions of pituitary activin on FSH secretion are presumably regulated by systemic inhibin and perhaps local follistatin, the differentiative actions of activin observed in ovarian cell culture may be closely regulated by locally derived follistatin to the exclusion of inhibin.

Using a different approach to study possible intraovarian actions of activin, Woodruff and colleagues (58) made the intriguing and unexpected observation that ovarian intrabursal administration of activin to immature rats caused follicular atresia. When injected in conjunction with systemically administered PMSG, activin prevented the development of healthy follicles characteristic of the follicle growth-promoting actions of PMSG. Intrabursal injection of inhibin, in contrast to activin, promoted development of healthy follicles similar to the effects observed following systemic administration of PMSG.

Evidently, the *in vivo* effects of activin appear to suggest a detrimental role for activin on the follicle, as opposed to its differentiative role inferred from the *in vitro* data. In an attempt to reconcile these divergent data, one obvious issue pertains to the different experimental systems used for study. In this regard, it has been reported that while estrogen treatment of immature rats promotes development of follicles to the preantral stage, many of these follicles are undergoing some degree of atresia (105). Consequently, the examination of granulosa cell function *in vitro* using cells obtained from estrogen-treated, immature rats has been called into question (105). On the other hand, the physiological implications of the *in vivo* approach used by Woodruff *et al.* (58) may also be questioned, in that the atretic effects of activin observed following injection into an immature rat ovary that contains only follicles in early stages of development may not accurately reflect the actions of this dimer in an adult ovary that contains corpora lutea in addition to a heterogenous population of both healthy and atretic follicles, all of which could modify the direction of activin's actions perhaps via the secretion of other growth factors (i.e., transforming growth factors, insulin-like growth factors, and epidermal growth factor). Alternatively, the follicular response to activin may be determined by the degree of exposure to gonadotropins. Hence, it would be interesting to determine the effects of intrabursal injection of activin on follicular development 1 day after (rather than in conjunction with) PMSG administration, when the ovary has been adequately exposed to gonadotropins. Regardless of the seemingly contradictory implications as to the intraovarian role of activin emanating from these reports, it is certain that future studies aimed at resolving this issue will yield important new information regarding the nature of the local control mechanisms governing the processes of follicular recruitment and atresia.

While additional *in vitro* and *in vivo* data should help elucidate the intricate roles of activin, as well as of follistatin, in regulating folliculogenesis, detailed *in situ* hybridization, histochemical, and immunohistochemical analyses of inhibin subunit and follistatin mRNA and protein in the ovary of cycling rats have already been conducted and may provide pertinent clues to the potential importance of an intraovarian activin-follistatin regulatory system. Again, bearing in mind that subunit gene expression and protein does not in itself constitute conclusive proof of activin production, results from histochemical studies have revealed that β-subunit mRNA and protein are localized exclusively to the granulosa cells of healthy follicles and show cyclic fluctuations according to specific days of the estrous cycle (66, 106). In general, variations in the expression of both inhibin-β subunit genes correlated with the intensity of staining of the respective subunit protein. On a developmental basis, β-subunit mRNA and protein were first observed in late secondary and tertiary follicles early on estrous morning, presumably as a result of the secondary FSH rise, since PMSG treatment of immature rats activates β-subunit gene expression in secondary follicles (67). β-Subunit protein and mRNA levels then are maintained through proestrous morning,

after which pronounced decreases in β-subunit mRNA and protein occur during proestrous afternoon coincident with the morphological and functional changes associated with luteinization of granulosa cells heralded by the preovulatory discharges of LH and FSH.

In a recent study conducted by our laboratory, both follistatin mRNA and protein, like the β-subunit cell products, were localized exclusively in the granulosa cells (74). However, several differences were noted between the expression of follistatin and inhibin-β subunit genes in the rat ovary during the cycle. Most notable was the detection of follistatin mRNA in healthy, as well as atretic, follicles. Importantly, whereas the signals for the β-subunit message and protein decrease between proestrus and estrus, the signals for follistatin mRNA and protein and, in particular, for the mRNA remain intense. Finally, follistatin gene expression and translation of mRNA transcripts do not appear to be coordinately regulated; whereas follistatin mRNA is present in healthy and atretic follicles, follistatin protein is detected only in healthy follicles. Despite these differences, one should be cautious in assigning physiological import to these differences pending a detailed analysis of cyclic fluctuations in inhibin-subunit and follistatin mRNA and protein levels performed on the same or adjacent sections of ovary by the same laboratory, since methodology and subjective analysis of data could differ between labs.

How then might histochemical data help resolve the conflicting *in vitro* and *in vivo* data reviewed earlier? Unfortunately, interpretation of histochemical data almost always evokes multiple scenarios that often results in further complication of an already complex situation. Regardless of whether one aligns oneself with the implications of activin's effects *in vivo* or *in vitro*, it is almost certain that the ability of activin to promote granulosa cell differentiation or follicle death is likely dependent on a ratio of follistatin to activin in the granulosa cell microenvironment. Thus, by virtue of its ability to bind activin, it could be inferred that follistatin might offset the atretic actions of activin. However, an intraovarian system solely between activin and follistatin for regulation of atresia, while plausible in theory, may be difficult to demonstrate experimentally since inhibin, like follistatin, can seemingly counteract the process of atresia by promoting development of healthy follicles (58).

Concluding Remarks

In the preceding discussions, we hope we have presented some provocative evidence to suggest the existence of an intrinsic regulatory system between activin and follistatin that acts to modulate tissue-specific functions. Much of the evidence stems from tissue localization studies, which, as mentioned previously, are open to a great deal of speculation concerning the potential physiological functions and interactions of the two proteins. To date, none of the evidence clearly establishes that the system is an integral component of cellular homeostasis. Nevertheless, an array of queries and issues arises from this research that deserve comment. For one, how and where does follistatin interact with activin? This is a very pertinent question, the answer for which likely will vary depending on the tissue. Furthermore, since some of the actions of activin, particularly those exerted on the gonadotrope and granulosa cell, are likely autocrine in nature, it is imperative to determine whether these autocrine actions are exerted within the cell interior and/or externally after secretion of activin. Such data will aid in our understanding of the actual site of interaction. In addition, a recent report shows binding of follistatin to granulosa cell-surface proteoglycans, a finding which will undoubtedly impact on the kinetics of interaction between follistatin and activin, at least in the ovary (107).

In this review, we have concerned ourselves with only the possible existence of a local system modulating folliculogenesis and hormonogenesis in differentiated organs after birth. In light of the actions of activin on embryogenesis and the turning on of β-subunit genes at critical points during this process (19–22), one might ponder whether activin and follistatin interact on developmental processes prior to birth. Very recent evidence suggests that such an interaction may be of functional significance prior to birth, since the expression of follistatin mRNA was found to occur at the same stage of *Xenopus* embryogenesis that β_B-subunit mRNA is first detected (21, 108). Consequently, the interaction of these two proteins may be important for a number of growth and differentiation processes prior to and after birth.

On a different note, whereas the detection of mRNA transcripts for both follistatin and the inhibin-β subunits in a wide variety of tissues provides a major impetus to suggest a functionally important regulatory system between follistatin and activin, the data in Table II clearly reveal that there are tissues in which mRNA for only one of these proteins is detected. Thus, this would suggest that follistatin may possess activities in these as well as in other tissues that are independent of its binding to activin, thereby leading one to predict the existence of bona fide receptors for follistatin. In addition, these data also would suggest that some actions of activin are not tightly regulated by binding to follistatin.

While it is quite obvious from previous data that gonadotropins are major activators of follistatin and β-subunit gene expression and translation (62, 67, 107, 109), we would like to impart one final comment pertaining to an intriguing hypothesis concerning the potential for androgens to be common stimulators of activin production in the AP and ovary. For many

years, androgens have been implicated as causative factors in promoting atresia (110–112). Likewise, androgens have been shown to selectively stimulate basal production of FSH under both *in vitro* (113) and *in vivo* (114) conditions. Moreover, increasing serum testosterone levels during proestrous afternoon have been shown to exert a modulatory role on the extent of FSH secretion during the secondary FSH surge (115, 116). Consequently, if it is assumed that activin has intrinsic atretogenic activities coupled with its characteristic ability to stimulate FSH secretion, then one could easily envision that the atretic and FSH-stimulating actions of androgens may well be mediated through stimulating the synthesis of β-subunit dimers.

What began as a search for an FSH-suppressing protein has now resulted in a new frontier, not only in reproductive biology, but in other areas of biology as well. As with other revolutions in biology in its formative stages, we cannot fully comprehend the significance of these peptides in all areas of biology. Part of this is attributable to the technological advances of our time that allow the identification of compounds before we can assign a biological role to them, which is in direct contrast to earlier times, when the biological significance of compounds were known before their identification. It is anticipated that the reader, regardless of scientific interest, will come away from this review with an air of both astonishment at the vast array of functions already assigned to activin and enlightenment at the potential importance of the follistatin-activin relationship to many areas of biology.

Note added in proof. Since the submission of this review, Kaiser *et al.* (73rd Annual Meeting of the Endocrine Society, Washington, DC, 1991 [Abstract 1131]) reported the detection of follistatin mRNA in a subpopulation of rat pituitary gonadotropes. Consequently, this finding suggests that, similar to the rat ovary, activin and follistatin are produced by the same cell type in the rat anterior pituitary gland, thereby buttressing the case for an intrinsic activin-follistatin system within gonadotropes for regulation of FSH biosynthesis and secretion.

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