MINIREVIEW

Activins, Inhibins, and Follistatins: Further Thoughts on a Growing Family of Regulators[1] (44130)

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Abstract. **Inhibin, a feedback inhibitor of pituitary FSH secretion, and its homodimer, activin, have been the subject of a growing body of literature in the last 5 years. These factors play a role not only in endocrine feedback in the reproductive system but also in paracrine and autocrine regulation of both reproductive and nonreproductive organs, including the liver, kidney, and brain. Additionally, the messages coding for both subunits and their receptors are exquisitely regulated, both spatially and temporally, during embryonic development. The cloning of a family of activin receptors; the development of specific immunoassays for inhibins A and B, and activins A and B; the description of α subunit, β subunit, and receptor loss of function transgenic mouse models; and the cloning of two new β subunit homologs have increased our understanding of the possible roles this complex family of proteins plays in development and endocrine function. This review largely confines itself to the roles of inhibins and activins in the male and female reproductive system, and is intended as an update to a 1992 review published in this journal.** [P.S.E.B.M. 1997, Vol 215]

Inhibins are a family of dimeric proteins whose mature form consists of an α and a β subunit (αβA or αβB). Activins are the homodimers (βAβA, βBβB) or heterodimers (βAβB) of the β subunits. Both the proteins and their receptors are included in the transforming growth factor–β (TGFβ) superfamily of proteins and receptors. Whereas inhibin was described as a feedback regulator of reproductive function many years ago (1), it has been just a decade since the cloning of the genes coding for these molecules (2). In that time there has been an explosion of information on the distribution, activities, binding proteins, and receptors for these factors, which play an important role, not only in reproduction, but in development and nonreproductive functions in the adult. This review will largely confine itself to the roles of inhibins and activins in the male and female reproductive system, and is intended as an update to a 1992 review (3) published in this journal.

The inhibin/activin family of hormones, while originally described as gonadally produced feedback regulators of pituitary hormone release, are now known to have broader-ranging effects both within and outside of the reproductive system. Inhibin has been described as a tumor suppressor, while activin has been shown to act, in a number of tissues, as a mitogen, motogen, and morphogen. In addition to acting as feedback inhibitors from the gonads to the pituitary, they also have significant paracrine, and autocrine, regulatory effects within the pituitary (3), ovary (4), placenta (5, 6), prostate (7), and testis (3). Activins, especially, seem to play an important role as morphogens in several species from xenopus (8, 9) to mouse (10). In addition, activin has been shown to be an antagonist of interleukin-6 (IL-6) and IL-11 (11). Its roles in erythropoesis

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(12), wound repair (13), and the regulation of bone morphogenesis (14) continue to be actively explored. Activin seems to play a critical role in the development (9, 15), function (16, 17), and response to ischemic injury (18) in the brain. It has also been shown to be present in, and to regulate the function of, the lung (19, 20), heart (21), vasculature (22), and liver (23, 24).

In contrast, the inhibin α subunit is much less widely expressed in the embryo than in the adult. The α subunit is expressed in the brain, adrenals, and gonads in the adult; in the gonads during early embryonic development in the rat (15, 25) and mouse (26); and in the adrenal and testis in the human embryo (27, 28). Transgenic animals made null for the inhibin α gene develop tumors in the gonads and adrenals (29, 30), suggesting a role for inhibin in both of these organs (31).

The availability of loss-of-function (''knock-out'') mutant mice with the deletion of the follistatin (32), inhibin α (29), activin receptor (type IIA) (33), activin βA (34), activin βB (35), or both βA and βB (33) genes has proven tantalizing in providing answers, and raising fresh questions, as to the physiological role of this family of growth factors in general, and in the reproductive system in particular. All of these animals develop to parturition, although several (the βA, βAβB, Act RII, and follistatin knock-out homozygotes) die at or soon after birth. One important question raised by these animals is how the loss of proteins whose mRNA and receptor expression (15, 36) is so exquisitely regulated during embryonic development can have such a seemingly minor effect on development.

Inhibin/Activin Family: A Tale of Increasing Complexity

The already complex inhibin family has become even more complex with the discovery using degenerate polymerase chain reaction (PCR) cloning methods, of two new genes in the β subunit family. The activin βC gene was cloned from a human liver cDNA library. All nine of the cysteines are conserved, and the predicted amino acid sequence has 53% identity with the mature human activin βA and 51% identity with the mature human βB (37). This activin seems to be expressed exclusively in liver in the mouse and might thus be important in paracrine regulation in this organ. A βD activin cDNA has been cloned from Xenopus with a 63% identity between the predicted amino acid sequences of the βC and βD mature proteins (38) (for comparison, the βA and βB subunits have 64% identity). Again, all nine cysteine residues are conserved. Both activin C and activin D are made as high molecular weight pro-forms with cleavage sites which could generate a mature form. The pro-forms both contain glycosylation sites. To date, neither protein has been purified. However, activin-like activity of activin βD has been demonstrated by injecting the βD mRNA into Xenopus embryos, resulting in the induction of a secondary body axis and the induction of dorsal mesoderm genes (38). It therefore seems reasonable to assume that these two genes do produce active proteins *in vivo* and that the activin family now consists, minimally, of four homodimers and, potentially, six β subunit heterodimers, while there are potentially four inhibins. Further studies on the co-expression of the α and four β subunits, and efforts to purify the proteins will help clarify how many forms actually do exist, and in which species and tissues.

In addition to the increased complexity at the genetic level, the evidence for numerous, biologically active forms of inhibin continues to increase (39–41). There are up to eight active forms of inhibin, with molecular weights ranging from 32 to >100 kDa, in both bovine and human plasma, in addition to the biologically inactive (on the follicle-stimulating hormone (FSH) release assay) 30-kDa pro-α N cleavage peptide released from the α-chain of inhibin during processing. While the exact forms made may vary from species to species and from tissue to tissue, it is clear that multiple forms have biological activity. Assessing their comparative activity remains difficult, however, since there is a potential for conversion of high–molecular weight forms to lower–molecular weight forms during bioassay and the different molecular weight forms are clearly recognized differently by different antibodies (40, 41). Activin, too, can be found circulating as a higher–molecular weight pro-form and as a monomer (39).

The α subunit is also present both in its monomeric pro-form and as the fragment resulting from the cleavage of the pro-form. These proteins are inactive in the pituitary FSH-release bioassay for inhibin. However, in some tissues and reproductive tract tumors, this inhibin α subunit may be the major form of inhibin present. This has presented a major challenge for the devising of specific immunoassays, as will be further discussed below. It is still an open question as to whether the inhibin α subunit itself has any biologic functions within the reproductive system (42).

The main biological question raised by the multiplicity of forms is, Are there substantive and meaningful differences in the tissue specificity, binding to binding proteins, plasma clearance, or activity of these forms, or are they irrelevant to biological function? In any case, they impact how data from immunoassays are gathered and interpreted. It is important to understand which subset of the complex array of inhibins is being recognized in any given assay and whether the antibodies and assay configuration used under- or over-report any of these forms relative to the standard used in that assay.

Binding Proteins: To Have and To Hold

Two classes of binding proteins exist for the activin/inhibin family of proteins: follistatins (for review see Ref. 43) and α_2-macroglobulins (3, 44, 45). Both of these proteins are produced in the gonads and are present in blood. Activins and inhibins bind more strongly to follistatins than to α_2-macroglobulin (44, 46). However, the concentration of α_2-macroglobulin in serum is so great compared with that of follistatin that α_2-macroglobulin seems to be the major

binding protein for both activin and inhibin in the circulation (44), with only a minor proportion of activin bound to circulating follistatin. α_2-Macroglobulin–bound inhibin and activin remain longer in the circulation than unbound forms (44, 47, 48). In addition, α_2-macroglobulin is present in the circulation in both a native and active conformation. The active conformation has a higher affinity for activin, but this complex is cleared from the circulation faster than the native α_2-macroglobulin/activin complex (49). α_2-Macroglobulin does not interfere with most activin or inhibin immunoassays (50–52), although some exceptions may exist. α_2-Macroglobulin does not neutralize the bioactivity of activin or inhibin in any system tested to date (44).

Follistatin is a family of proteins generated by differential splicing (288- and 315-kDa forms) and proteolytic processing (303-kDa form) (53). These forms display differential heparin binding affinity, and therefore differential affinity for cell surface binding, with the 288-kDa form having a higher heparin binding affinity. Recent work suggests that the two forms may also bind activin in a different fashion (54).

Follistatin acts to inhibit activin activity in most, but not all, biological systems studied. It inhibits FSH release by pituitary cells *in vitro* and inhibits the ability of activin to stimulate K562 differentiation *in vitro* (44). It inhibits activin-stimulated granulosa cell division and follicle formation in ovarian granulosa cells *in vitro* (55). In contrast, while follistatin inhibits activin-induced Sertoli cell reaggregation *in vitro,* it does not inhibit activin-stimulated germ cell proliferation in the same co-culture system (56), suggesting more than one mode of action of activin in these two cell types. Follistatins have been shown to inhibit the binding of activin to the activin type IIA, IIB2, and IIB4—and, as a consequence, to the type I—receptors expressed in COS cells *in vitro* (57). Cell surface–bound follistatin does not seem to act to present ligand to signaling receptors in this system. The high binding affinity of follistatin for activins may also present serious interference problems in activin immunoassays (51).

Follistatin can also bind inhibin, although at a much reduced affinity compared with activin. It has not been possible to assess the effect of follistatin on inhibin bioactivity due to the presence of endogenous activin in the pituitary assay and the lack of effect of inhibin alone in the K562 assay. Follistatin does not interfere with a number of inhibin immunoassays (41). It is not known at this time whether the different forms of activin and inhibin have different affinities for the follistatins.

Receptors: Actions and Interactions

It is now apparent that the activin receptors are part of a family of related receptors which bind members of the TGFβ family of ligands. This superfamily has recently been reviewed elsewhere (58). Briefly, there are two types of activin receptors designated type I and Type II. The known type I receptors include ActRI (also called ALK-2) and ActRIB (ALK-4). Related type I receptors (e.g., ALK-1, B1) bind both TGFβ and activin (59, 60). The type II receptors are ActRII (61, 62) and ActRIIB. Several splice variants of the mammalian ActRIIB receptor exist (63). Other related receptors bind TGFβ, the bone morphogenic proteins (BMPs), and the drosophila *daf* proteins (63, 64).

Both the type I and type II receptors are serine/threonine kinases and were the first such receptors identified in vertebrates. The type II receptors bind to the type I receptors, and this complex tightly binds activin and elicits downstream signaling through phosphorylation of proteins, including the receptors themselves, by the integral kinases (65–67). The type I receptors are known to be unable to bind activin alone, while the type II receptors can bind activin. The type I receptors are phosphorylated by the type II receptor:activin complex. Kinase-deficient type II receptors fail to phosphorylate the type I receptor and act as dominant negative mutants, blocking activin-induced transcriptional activity (62, 68, 69). Kinase-deficient type IB receptors also block transcriptional activation of the receptor complex by activin, but this is not true of kinase-deficient type I receptors, indicating that the two are functionally distinct (69). Thus, the association of a type I and a type II receptor with an activin ligand, as well as the phosphorylation of both the type I and type II receptors, is necessary for signal transduction.

The relative affinity of activin A and activin B for the various possible combinations of the several type I and type II receptor complexes is not known. The activin type II receptors described above will bind inhibin, but with a lower affinity than activin (61). However, the relative affinity of inhibin and activin for this complex cannot explain all the physiological effects of inhibin. In addition, direct binding of inhibin to both isolated cells (70) and tissue slices in the testis (71) and ovary (72) indicates that there are cells in the reproductive organs that bind inhibin but do not bind activin. In contrast, *in situ* ligand binding of brain tissue slices (73) revealed binding of activin with minor competition by an excess of inhibin, as would be expected for the receptor complexes discussed above. These data suggest that a separate inhibin binding receptor(s) or component(s) remains to be discovered.

New Assays: New Ideas and a Reassessment of Old Ideas

At the time of our last review in 1992 (3), there were relatively few assays available to assess biologically relevant levels of the inhibins and activins. Great progress has been made in this area within the last few years. Several immunoassays now exist which measure the following: all inhibins (the *ck/ck* assay) (50), all inhibin A forms including the α subunit (Monash RIA) (74, 75), dimeric inhibin A only (50, 52, 76), dimeric inhibin B only (77–79) free activin A (51), "total" activin A (80), activin B (51), the α subunit only (81, 82), and the follistatins (83, 84). With this wealth of assays comes the need to relate results obtained

with different assays to each other. The characterization and availability of an international WHO inhibin standard (85) should be a great help in this regard. Additionally, Robertson *et al.* (41) have characterized six of the different inhibin assays as to their ability to detect the different forms of inhibin found in human follicular fluid, IVF serum, and male plasma. These were compared in their quantification of, and cross-reactivity with, purified recombinant inhibins and activins, and two pituitary bioassays. The pattern of the forms detected was similar in all three monoclonal sandwich assays and the bioassay. In contrast, all three polyclonal-based assays detected relatively more small–molecular weight components (probably including the 29K pro-α C).

While the forms of inhibin As detected and the cross-reactivities with other family members were similar for the three polyclonal assays, the absolute amount of inhibin A measured relative to the same recombinant human (rh-) standard varied considerably (e.g., from 50 to 160 ng/ml in the 55K fraction). The polyclonal assays varied even more in the absolute levels of "total inhibin" reported. One interesting and somewhat unexpected observation was the low cross-reactivity (<17%) of most of the "total" inhibin assays with inhibin B. Only the *ck/ck* polyclonal assay had considerable cross-reactivity (82%) with rh-inhibin B. Additionally, the ovine and rat pituitary bioassays showed considerably different abilities to detect rh-inhibin B, with the ovine assay being 20-fold less sensitive (41).

The use of specific inhibin B assays to measure inhibin B in the plasma of women during the menstrual cycle (78) yielded the surprising result that, while inhibin A and inhibin B reach similar levels (approximately 100 pg/ml) in the plasma, there is an inverse temporal pattern of secretion of the two inhibin forms with inhibin B high before the mid-cycle LH peak and low after, and inhibin A low before the LH peak and high after. Similarly, while inhibin A rises during pregnancy and remains high through the first 2 months, inhibin B falls at conception and remains very low (77). These data suggest that inhibin A and inhibin B have distinct biological roles in regulating ovarian function.

Inhibin A is present in very low amounts (<2 pg/ml) in male plasma (41, 79), and many studies have failed to find a good correlation between testicular function and FSH and inhibin A levels (1, 86). In contrast, inhibin B is present in substantial levels (average of 180 pg/ml) in normal male plasma (79). There is also a strong inverse correlation between circulating FSH and inhibin B in infertile men with elevated FSH, suggesting that inhibin B may be the major circulating inhibin in the human male.

Inhibin, Activin, and Follistatin in the Testis

Inhibin is historically first associated with the testis (1). After more than six decades of research, we are still learning about the role of this interesting family of peptides in testicular development, function and pathology. The importance of the inhibin family of peptides has been underscored recently by the knock-out technology employed by Matzuk and colleagues (29, 30, 32–34, 87) and recently reviewed (88).

Production of Inhibin Peptides in the Testis.

Transcriptional regulation. Inhibin α subunit gene expression is detected predominantly in Sertoli cells and appears to be regulated by FSH acting *via* cAMP. The cloned sequence from the 5′-flanking DNA from the α gene revealed the presence of several cAMP response element (CRE)–like sequences. In rat granulosa cells, only one of these was found to be functional (89), and such studies have not yet been done in Sertoli cells. However, it is clear that FSH and cAMP can increase α mRNA levels in rat Sertoli cells, mediated both by stimulation of transcription rates and stabilization of mRNA transcripts (90–93). Similarly, the bovine α inhibin gene was found to contain a cAMP response element (94), and it has been shown in the human testis that inhibin α subunit mRNA may be regulated *in vivo* and *in vitro* by human menopausal gonadotropin, and *in vivo* by hCG (95).

Additional regulation of the inhibin α subunit gene may be afforded by negative regulatory DNA elements in the upstream region of the gene, as shown in testicular MA-10 cells (96). It has also been demonstrated that the 5′-proximal sequence of the inhibin α gene contains DNA elements important for testicular expression in a cell type–dependent manner. A 2.5-kb section of the 5′-flanking region (promoter/enhancer element) of the mouse inhibin α gene directed transgene expression in immature and adult Sertoli cells, and also sporadically but strongly in Leydig cells (97). Photoperiod regulation of α subunit mRNA independent of FSH levels has been shown in the Siberian hamster, but the molecular effectors of this response are unknown (98).

It is thought that the major form of inhibin secreted by Sertoli cells is inhibin B (79, 99–101), in which case it might be expected that the βB subunit mRNA would also display FSH-mediated regulation. Namiki *et al.* (95) found no regulation of human testicular βB mRNA expression by either hMG or hCG *in vivo* or *in vitro*. However, βB subunit mRNAs are transcribed from different initiation sites (4.8- and 3.7-kb mRNAs), and although the βB gene does not have a classic CRE (99, 102), it is thought that the promoters, which contain putative AP-2 sites, may be controlled by upstream regulatory elements (103). Indeed, a cAMP response was observed in primary cultures of rat Sertoli cells, and TM.4 cells, in which βB mRNA expression was stimulated by cAMP via transcriptional induction (91).

The expression patterns of both the α and βB mRNA subunits are stage dependent (104) (see below). If the expression of the subunits is highly regulated by FSH as discussed, and FSH is known to have stage-dependent sensitivity (105), is the pattern of subunit expression secondary to the stage-dependent responsiveness to FSH? Kaipia *et al.* (106) found by Northern blot analysis of rat seminiferous tubule segments *in vitro* that FSH stimulated inhibin α

mRNA equivalently in stages II–VI and VII–VIII, even though FSH responsiveness is lower in stages VII–VIII. Inhibin βB mRNA was unaffected by FSH treatment under these conditions. It was concluded that elements other than FSH sensitivity account for the cyclical expression of the inhibin subunits in the seminiferous epithelium. In fact, alternative paracrine control of inhibin subunit mRNA expression has been demonstrated in rat Sertoli cells *in vitro*. For example, basal and FSH stimulated α subunit message were enhanced by TGFβ1 and activin A, and were decreased by tumor necrosis factor–α (TNF-α). βB message levels were unaffected by TGFβ1, but were decreased by TNF-α (107).

Localization of inhibin and activin production in fetal and prepubertal testis. Inhibin subunit mRNA and/or peptide has been detected from early in development in many species. Immunoreactive α and βB subunits were observed in seminiferous epithelium of fetal rats (100), and βA and βB mRNA was present in rat testes from 15-day post coital embryos onward (108). During mid and late gestation, high concentrations of inhibin are found in the testes of sheep and cattle fetuses (109). α subunit expression, by immunocytochemistry and *in situ* hybridization, was observed in the developing seminiferous cords in fetal sheep testis from Day 100 of gestation onward. Low levels of βA mRNA were also observed, but the protein could not be detected by immunochemistry. A proportion of Leydig cells were positive for immunoreactive α subunit throughout fetal development (110). Immunoreactive inhibin was secreted by both Sertoli and Leydig-like cells from 18-day-old chicken embryos, and could be stimulated by both FSH and LH (111). In the human, immunoreactive subunits were present in midgestation fetuses. All three subunits were present in interstitial cells, but only the α subunit was detected in Sertoli cells. In late-gestation rhesus monkey testis, the α subunit in Sertoli cells was accompanied by βB, but not βA (101).

In neonatal rats, immunoreactive α and βB are colocalized in the Sertoli cells, and βA has been found in the nuclei of immature germ cells at the periphery of the tubule (in pachytene and zygotene spermatocytes). βA was also observed in the testicular interstitium (100). In the Djungarian hamster testis, intense immunoreactivity for the α subunit was observed in prespermatogonia at Day 0 and decreased gradually to undetectable levels by Day 15. The α subunit immunoreactivity then increased in spermatogonia and Sertoli cells, with strongest staining at stages III and IV. However, dimeric inhibin could not be detected by immunoblot. Rather, it was concluded that prespermatogonia produced mostly monomeric α subunit precursor pro-$\alpha_N\alpha_C$, and mature Sertoli cells and A spermatogonia mostly $\alpha_I\alpha_C$ (112). In a similar pattern, the α inhibin gene is upregulated in the Sertoli cells of sheep and bull testes in the prepubertal gonad, and then downregulated in early puberty (94). High testicular immunoreactive inhibin levels have also been observed in humans in the first few months of life (113).

It therefore appears that in the developing testis the proliferating Sertoli cells readily express α subunit, which is most likely ultimately coupled to the βB subunit to form inhibin B as the Sertoli cells mature. However, in some instances free α subunit is secreted alone. Additional support for this is illustrated by the effects of hypo- and hyperthyroidism in the rat. After pharmacologically induced neonatal hypothyroidism, Sertoli cell proliferation is prolonged. As a result, α subunit expression is unaffected, but expression of βB is delayed (as was ABP) (114). Conversely, high neonatal triiodothyronine levels reduced the period of Sertoli cell proliferation; inhibin α immunoreactivity was high on Days 5–9, but low on Days 16 and 23 (115), suggesting inhibin protein levels may be indicative of the maturation and differentiated state of the Sertoli cell population. This would be expected if inhibin acts as a feedback inhibitor of FSH, which is required for Sertoli cell development in the male.

Localization of inhibin and activin production in the adult testis. In the adult it is apparent that there is more interspecies variation in the patterns of subunit expression, and this may reflect the stage-dependent expression of these proteins in the mature seminiferous tubule. In the adult rat, all subunits are expressed in Sertoli cells, and therefore the potential exists for secretion of any combination of the mature peptides. For example, in addition to the secretion of inhibin and free α subunit, de Winter *et al.* (116) have shown that rat Sertoli cells *in vitro* secrete both immuno- and bioactive activin A. In the rat, the α and βB subunits are expressed at their greatest levels at stages VIII–XI and XII–V, and are evenly distributed in the Sertoli cell cytoplasm (100, 104, 117). βA mRNA expression is highest in stages IX–XI, in which the pattern of expression is concentrated in the Sertoli cell cytoplasm around leptotene spermatocytes (104).

Among four nonhuman primate species and men from 31 to 85 years of age, inhibin α and βA were present in the cytoplasm of Sertoli and Leydig cells, with none in the germ cells. In this case, no pattern according to stage was observed. Leydig cells contained both the α and βA subunits or the βA alone, and the βA expression in Sertoli cells of older men was more intense. Additionally, the signal for βA was very intense in normal and hyperplastic human Leydig cells (118). Within the Sertoli cells, the α subunit in adult humans was localized by electron microscopy to the Golgi cisternal spaces, rough endoplasmic reticulum (RER) (suggesting protein synthesis) and coated vesicles in the cytoplasm. α subunit was also present in coated pits and vesicles of some spermatocytes, which may be indicative of uptake. α subunit has also been localized in the RER of Leydig cells (119).

The pattern of expression within the seminiferous epithelium in adulthood suggests a purposeful and intimate communication between Sertoli cells and germ cells at various stages of development. Indeed, in the human it has recently been shown that immunoreactive inhibin production from Sertoli cells is stimulated in the presence of germ

cells (120), complementing previous observations of this nature in the rodent (121–123).

The pattern of production in the interstitium is becoming more clear, owing to a better understanding of the contributing cell types in the intertubular spaces, and the differentiative pathways of these cell types. In 1989 it was demonstrated that interstitial cells could secrete activin (90). Bioactive activin was secreted by interstitial cells from the immature rat and pig, and βA and βB mRNA could be detected in the absence of α subunit in the pre-pubertal Leydig cell–derived line TM.3. The latter was confirmed, together with the detection of activin A by immunocytochemistry in TM.3 cells, by Ying *et al.* (124). The α subunit mRNA could not be detected in interstitial cells of young rats but appeared in those of older animals (Krummen, Mather, unpublished data). It has been shown that inhibin and activin subunit genes are co-expressed in four cultured rodent tumor Leydig cell lines (125), although they do not appear to secrete bioactive activin, but rather secrete bioactive inhibin (126). Other labs have also reported that Leydig cells do not produce bioactive activin but do secrete inhibin α subunits (127, 128). The peritubular myoid cell, which separates the tubular and interstitial compartments, expresses high levels of inhibin βA subunit mRNA (and some inhibin α and βB mRNA), and secretes both immuno- and bioactive activin A (129). At this time, it is not known whether this secretion is directional towards the tubular or interstitial compartment. Activin and inhibin secretion may thus occur in different cells in the testis in animals of different ages and in the adult Sertoli cell at different stages of the seminiferous cycle. These changes, and the level of follistatin secretion and receptor expression, would then determine the net biological activity that the cells are exposed to.

Receptors and Binding Proteins.

Activin receptors. Several types of activin receptor exist within the testis, all of which also bind inhibin, but at much reduced affinities. Activin receptor type II (ActRII) has been found to be expressed ubiquitously during testicular development, from Day 14 pc onward (108). In the adult ActRII was detected by Northern analysis of isolated rat testicular cells in pachytene spermatocytes, round spermatids, and some in Sertoli cells (130). Similarly, *in situ* analyses have detected message for the receptor in diplotene primary spermatocytes at stage XIII, dividing spermatocytes at stage XIV and in step 1–4 spermatids, with either low or no detectable signal in Sertoli cells (104, 131). Millar *et al.* (132) confirmed the identification of ActRII hybridization on round spermatids and pachytene spermatocytes but questioned the association with Sertoli cells observed at stages IX–X. It was suggested that this observation was an artifact, caused by riboprobes binding to residual bodies undergoing dissolution following fusion with Sertoli cell lysosomes.

ActRII has not been associated with Leydig cells by *in situ* hybridization; however, ActRII is expressed in four different Leydig cell tumor lines (96) and TM.3 cells (124). Activin receptor type II mRNA was also detected in cultured rat peritubular myoid cells (129).

ActRIIB2 was found to be the major isoform of the ActRIIB in adult rat testis; yet, by Northern analysis, levels of the type IIB receptors were much lower than those of ActRII mRNA (133). Expression of rat ActRIIB2 mRNA is most abundant at Day 15 pc, and then diminishes with time (108), although stage-dependent patterns of expression can be observed in the adult. ActRIIB2 mRNA was shown to be expressed maximally at stages IX–XI, basally in type A_1 and A_2 spermatogonia and in Sertoli cells. In the pubertal rat testis, the expression was localized in Sertoli cells around primary spermatocytes and meiotically dividing cells (134). However, Cameron *et al.* (131) found ActRIIB to be absent in tubules by *in situ* hybridization, and weakly expressed in interstitial cells. It is possible that this disagreement results from the nonspecific signal sometimes observed in stages IX–X, as discussed above (132). No studies on the expression of type I receptor mRNAs in the adult testis have been reported at this time. Since a type I/type II receptor complex is required for downstream signaling, this is an important area for further study.

Binding studies using [^{125}I]activin in rat testis (71) found binding in a non–stage-dependent manner to cells located in the basal compartment of the seminiferous tubules at all ages, and this was partially competable by inhibin. This could represent binding to ActRII or ActRIIB, to a mixture of both, or to membrane-bound follistatin. In adult rats (45 and 60 days old) activin bound in a stage-dependent fashion to spermatids in stage VII–VIII tubules, and this was not competable by inhibin. This is more likely to represent binding to ActRII, and it may be that functional binding is delayed relative to mRNA expression.

Inhibin receptor. The identity of the inhibin receptor has yet to be revealed; however, evidence exists to suggest that there is (at least) one to be found. Woodruff *et al.* (70) observed FITC-conjugated inhibin A binding to rat leptotene/zygotene spermatocytes. Activin A labeled in the same way did not bind to these cells, which do not exhibit ActRII or ActRIIB receptors. It has also been shown that [^{125}I]inhibin binds to 3 βHSD–positive rat testicular interstitial cells throughout development, from 15 to 60 days of age, and activin could not compete for these binding sites (71).

Follistatin. Follistatin clearly displays inhibitory properties on FSH release by the pituitary (facilitating its identification) and in the paracrine control of activin function in the testis (56, 135, 136). However, the phenotype of the follistatin knock-out is also a loss of function, perinatal lethal mutation, suggesting that more is yet to be learned about the role of follistatin and/or the effects of excess activin during development (32). Furthermore, since the effects of the follistatin knock-out were more widespread than those in the activin-deficient mice, this suggests that follistatin may be involved in the modulation of other protein activities in addition to those of the known inhibin and activin proteins (32).

In the male, in contrast to the female, follistatin expression cannot be detected by Northern analysis in fetal (Days 14, 15, and 18 pc) or prepubertal rat testis (108). In the adult, expression was detected in Sertoli cells around leptotene spermatocytes at stages IX–XI (i.e., at stages corresponding to βA and ActRIIB2 expression) (104). However, immunoreactivity could only be seen in nuclei of spermatocytes, spermatids, and Leydig cells, but not in Sertoli cells (137).

Follistatin mRNA expression in rat Sertoli cells could be regulated by serum, EGF, and activators of protein kinase C. Forskolin, PGE_2, and all-*trans* retinoic acid, all extragonadal inducers of follistatin expression, had no effect on the Sertoli cells. Similarly, activin, which stimulates follistatin expression in granulosa cells, had no effect on Sertoli cell follistatin mRNA levels, indicating that the regulation of follistatin is sex and tissue specific (43, 138). Follistatin also displays testicular cell specificity, as discussed above (56).

Activities of Inhibin Peptides.

Inhibin. The interpretation of the actions of inhibin and activin has been largely influenced by the recent employment of knock-out technology in this field. Inhibin α–deficient male mice survive embryonic life, are viable, and have normal overt sexual differentiation (29). Therefore, expression of inhibin in the embryonic gonad is not essential for the formation of mature germ cells or for normal sexual development. However, a previously unknown, but crucial, role for inhibin as an autocrine tumor suppressor protein became unmasked when it was observed that from 4 weeks of age, inhibin α–deficient mice developed mixed or incompletely differentiated gonadal stromal tumors. In the testis, there was no Leydig cell hyperplasia; in fact, a decrease in Leydig cell numbers was observed. However, in inhibin α and MIS double knock-out mice, Sertoli cell tumors developed accompanied by Leydig cell neoplasia (87). This suggests that inhibin and MIS have synergistic roles in the testis.

The physiology of the tumors in the α-deficient mice also provided information about the paracrine communication within the inhibin family of peptides. In the testicular tumors, a 200-fold increase in the expression of βA subunit mRNA was observed, together with a 3-fold decrease in the expression of ActRII (139). This suggests that inhibin may regulate activin either transcriptionally or post-transcriptionally. In addition, high levels of activin may downregulate its own receptor (139).

Previously, experiments *in vitro* have shown that inhibin could increase luteinizing hormone (LH)–stimulated steroidogenesis in immature Leydig cells (140). It seems feasible that inhibin may have a modulatory function on Leydig cells, since they appear to exhibit specific binding sites (71). Many locally secreted factors have the ability to modulate steroidogenesis, several of which originate in the Sertoli cell. In the α knock-out animal it is possible that alternative compensatory factors maintain testosterone secretion, especially in the presence of elevated levels of FSH (29).

It has also been suggested that inhibin can regulate spermatogonial number. Inhibin treatment caused a reduction in spermatogonial numbers *in vivo* in adult mice and Chinese hamsters (141), and inhibited DNA synthesis in adult rat tubular segments *in vitro* (142). It is possible that this function is entirely restricted to the adult animal, in which case subtle regulatory control of spermatogenesis may not have been observed in the α knock-out animals owing to the extent of the tumors in the adult mice. Alternatively, the reported effects on spermatogonia might not be mediated directly *via* inhibin, but rather *via* an inhibin-dependent reduction in activin, as suggested by the α knock-out. Recent α subunit immunoneutralization studies showed no effect on testicular morphology after treatment, in rats from birth to 25 days old (143), which agrees with observations in the α knock-out (88).

As previously discussed, it is widely believed that the major form of Sertoli cell secreted inhibin in many species is inhibin B. It is relevant, therefore, that the male βB knock-out mouse was fertile and viable (35, 144), suggesting either that the βA subunit can substitute efficiently in the systemic production of inhibin, or that the knock-out phenotype is specific to the mouse. It is difficult to disregard the apparently intricate expression patterns of the βB subunit in the seminiferous tubule.

Activin. Analysis of knock-out experiments of the activin-related subunits and receptors is more complex than that of the α knock-out because of the multiple types of receptors, and the early lethality of the βA knock-out phenotype (34). Obviously, the effects of the βA subunit are systemically wide-ranging, and so we await the phenotype of the temporally or tissue targeted deletion of the βA gene.

Activin has been shown to be a potent stimulator of spermatogonial proliferation, and perhaps survival, *in vitro* (135) (Moore & Mather, unpublished observations). Additionally, activin can induce tubule formation in mixed cultures of rat germ and Sertoli cells (135). Hakovirta *et al.* (142) observed increased DNA synthesis in rat intermediate spermatogonia (stages III–IV), and additionally in preleptotene spermatocytes (stage VII), following treatment with activin A *in vitro.* However, Kaipia *et al.* (108) found that activin inhibited thymidine incorporation into the fetal testis at Day 14 pc, although this effect was lost at later embryonic days.

In addition to modulating germ cell proliferation, activin also has mitogenic effects on the Sertoli cell. Together with FSH, activin stimulated Sertoli cell proliferation in 9-day-old rat testes (136), where FSH alone was insufficient. This synergism is similar to that seen in immature granulosa cells (see below). At 18 days old, no further proliferation could be induced. Neither activin nor FSH, nor a combination of both could not induce Sertoli cells to reenter mitosis once proliferation had ceased. These observations fit well with effects of ActRII knock-out. ActRII-

deficient mice were delayed in reaching fertility and had smaller testes from 21 days on (34). They displayed decreased seminiferous tubule diameter and total tubule volume, and reduced numbers of total Sertoli cells. Although the stages of spermatogenesis were essentially normal, the reduced fertility is a result of the reduction in tubule volume, leading to decreased spermatozoa. Adequate sperm quantity in adulthood is dependent on correct Sertoli cell number during development, so it is clear that activin contributes to the development of the adult Sertoli cell population, and that at least part of this effect is mediated *via* ActRII. However, spermatogenesis can occur despite reduced FSH and without signal transduction through ActRII. It is also important to note that activin can promote the *in vitro* growth of gonadal sex cord stromal tumor cell lines similar to those arising in the testes of inhibin α–deficient mice, with additional loss of p53 (145). Together, these observations indicate that, while the correct differentiative checkpoints are in place, activin can only stimulate growth of the Sertoli cell population until it is mature. However, upon loss of differentiative control, activin will readily promote the growth of undifferentiated cells, ultimately leading to tumor formation.

In addition to proliferative effects, activin may act on the mature Sertoli cell. Activin A inhibited FSH-stimulated aromatase activity and androgen receptor mRNA expression (basal and FSH stimulated) in rat Sertoli cells (116) but stimulated basal and FSH-stimulated inhibin and transferrin production in similar cultures (129).

Activin may also have effects in the testicular interstitium. The inability of activin to compete with inhibin binding to Leydig cells in the rat (71) and the demonstration of ActRII receptors on peritubular myoid cells suggests that some effects on steroidogenesis may be indirect, and may also account for some of the conflicting data in this area. It was originally shown that activin inhibits LH-induced steroidogenesis in whole testis cultures from the rat (140). In contrast, Ling *et al.* (146) reported a stimulation of testosterone release *in vitro* in response to activin.

Activin also reduced hCG-stimulated dehydroepiandrosterone (DHEA) accumulation in immature porcine Leydig cells, increased the conversion of exogenous DHEA and pregnenolone into testosterone *via* a reduction in $P450_{SCC}$ activity, and increased 3βHSD (147). While it is clear that these proteins can modulate steroidogenesis, more work needs to be done to elucidate the cellular and hormonal interactions involved in this regulation.

Intraovarian Regulation: Inhibin, Activin, and Follistatin

Inhibin/activin and follistatin were initially characterized as ovarian endocrine factors by their ability to regulate pituitary FSH release. The feedback regulation of FSH release by inhibin has been reviewed elsewhere (4). However, a growing body of evidence suggests that activins, and their binding protein follistatin, are at least as important as local paracrine/autocrine regulators as they are as endocrine feedback hormones. This section will review the evidence for the role of inhibins and activins in intraovarian regulation.

Local Amplification of Gonadotropin Signal. The expression of inhibin subunit mRNA and protein in the ovary has been reviewed by Woodruff *et al.* (4, 148). The level of expression and the type of subunit expressed in granulosa, thecal, and luteal cells changes with the stage of the estrous cycle. Numerous experiments have shown that gonadotropins, steroids, and activin itself directly stimulate the expression of inhibin subunits *in vitro* and *in vivo*. These facts provided the fundamental basis for the hypothesis that inhibin/activin may be local mediators of gonadotropin stimulation of folliculogenesis.

Specific binding sites for activin and inhibin have been identified in the ovary by *in situ* ligand binding (71). However, *in situ* binding of [^{125}I]-labeled ligands, especially activins, cannot differentiate receptors from binding proteins such as follistatin, which is largely responsible for the binding of activin in the follicular fluid. *In situ* hybridization of receptor messages provided additional valuable information for receptor localization. However, only very low levels of the known receptor mRNAs could be demonstrated in granulosa or thecal cells where activin responses have been clearly demonstrated (25, 131, 149). Using northern blot analysis, Erammaa *et al.* (150) reported human granulosa cells isolated from preovulatory follicles express all currently known serine/threonine kinase activin receptors.

Synergistic effect of activin and FSH on granulosa cells. In immature granulosa cells, FSH receptors can be induced by FSH itself given at low concentration. High concentrations of FSH, in contrast, will result in the downregulation of FSH receptors. Both the induction and downregulation of FSH receptors by FSH are enhanced by activins (151). In addition, activins were able to induce FSH receptors in granulosa cells in the absence of FSH (151, 152). In small follicles, where granulosa cells show little response to FSH due to the lack of FSH receptors in these cells, the initial induction of FSH receptor might then be attributed to activin (Li and Mather, unpublished data). The form of activin involved in this process is most likely activin B, since both inhibin βB subunit mRNA and βB homodimers have been found in small follicles in primates (101).

The rapid expansion of granulosa cell number in recruited follicles is gonadotropin dependent. However, FSH alone has little effect on the proliferation of granulosa cells *in vitro*. In contrast, activins stimulate the proliferation of granulosa cells in cultures from rat small follicles, large secondary follicles (55, 153, 154), and human granulosa-luteal cells from preovulatory follicles (101). FSH enhances the activin-stimulated cell proliferation in cells from large follicles but not in the cells from small follicles (55). Additionally, activin has been shown to stimulate the proliferation of sex cord cells during embryonic life, which will

give rise to granulosa cells at later stages of development (108).

This evidence suggests that activin is a common mitogen for the cells of granulosa cell lineage. It seems that FSH acts to promote granulosa cell proliferation by two different mechanisms. FSH acts indirectly by increasing activin levels in the follicle, which in turn stimulate granulosa cell division. FSH also directly enhances the activin-stimulated cell proliferation observed in granulosa cells from large follicles.

Regulation of steriodogenesis by inhibin and activin. The major effect of inhibin upon steroidogenesis in the ovary is the augmentation of androgen production. Treatment with inhibin augments luteinizing hormone-stimulated androgen production by rat (140), human (115), and bovine (156) thecal/interstitial cells *in vitro*. Since thecal androgen is vital as an aromatase substrate in the two-cell, two-gonadotropin mechanism of estrogen synthesis (157), augmentation of LH-stimulated androgen synthesis by locally produced inhibin would be expected to enhance follicular production of estradiol. Indeed, immunoneutralization of endogenous inhibin in rat ovarian follicle culture results in a significant decrease in estradiol secretion (158). As a competitor of inhibin, activin depresses the inhibin-stimulated androgen production in thecal cells.

While inhibin increases androgen production in thecal cells, activin enhances activity of the FSH-induced aromatase enzymes converting androgen to estrogen. However, the effect of activin on progesterone production changes with the stage of follicular development (55, 151, 153, 159, 160). In nondifferentiated granulosa cells, activin increases the FSH induced progesterone production by stimulating cholesterol side-chain cleavage cytochrome P450 mRNA transcription. However, it inhibits progesterone production in differentiated granulosa cells by reducing 3β-hydroxysteroid dehydrogenase, Δ^5/Δ^4-isomerase, and cholesterol side-chain cleavage cytochrome P450 mRNA transcripts (153). It therefore seems likely that locally produced activin promotes the initiation of folliculogenesis in small follicles but inhibits the luteinization of large antral follicles.

Follistatin as a Local Regulator. Follistatin was first identified in and isolated from ovarian follicular fluid. As a gonadal protein, it has been shown to suppress the biosynthesis and secretion of FSH, but not LH. However, it is questionable whether follistatin acts as an endocrine regulator, because the overall plasma follistatin level, measured by radio-immunoassay, does not fluctuate across the normal estrous/menstrual cycle (161, 162). Therefore follistatin more likely belongs to the category of local regulators of FSH at the level of the pituitary. In the ovary, the expression of follistatin is closely correlated with the expression of the inhibin β subunits and is similarly regulated by gonadotropin (163–165). As an activin binding protein, follistatin has been shown to neutralize activin activity on ovarian cells *in vitro* (55, 151, 159, 166). However, since its neutralizing activity is dependent on the follistatin/activin ratio, as demonstrated in testicular cultures (56), follistatin may act as a trap to keep locally produced activin from leaving the follicle and ensure the dominance of the follicle. The high-affinity binding of follistatin to the extracellular matrix through its heparin-binding domain would make follistatin well suited to function in this manner.

Folliculogenesis and Atresia. FSH is a premier hormone in regulating folliculogenesis. Injection of FSH induces superovulation, and, conversely, withdrawal of FSH by passive immunization or hypophysectomy results in massive atresia. After small follicles have been recruited into the growth pool by the secondary FSH surge, the growing follicles produce steroids and inhibins to negatively regulate FSH synthesis and secretion. Those follicles capable of withstanding the declining FSH concentrations become dominant follicles, while others undergo atresia. Dominant follicles must compensate for the declining FSH by producing local factors that can be retained in the follicle and are able to amplify the FSH signal. Activin fits both of these criteria and therefore is a potential candidate for playing a major role in follicular selection.

While results of the *in vitro* experiments discussed above support this model, several *in vivo* results deserve discussion. Local administration of recombinant activin to immature, PMSG-injected rats caused widespread atresia in maturing follicles (167). Such a level of atretic response has often been observed following gonadotropin withdrawal or desensitization by a supraphysiological dose of gonadotropin. It is possible that local intrabursal administration of a large dose of activin may turn the hyperstimulating dose of PMSG into a desensitizing dose. More recently, daily intravenous injections of activin have been shown to increase the number of large follicles and induce premature ovulation in the rat (168).

Expression of Inhibin Subunits in Disease Models. Recent advances have been made in our understanding of physiology of the inhibins by using disease models. In hypophysectomized rats, Aloi *et al.* (169) demonstrated that FSH-regulated inhibin subunit mRNA expression required the presence of LH. An analysis of the inhibin subunit expression during early postnatal development (Day 1 to Day 15) in normal and hypogonadal mice (containing a deletion in the gene encoding GnRH) demonstrated that postnatal development occurs in the absence of detectable circulating gonadotropin (170). They concluded that normal expression of β subunits was completely gonadotropin dependent around the period of late primary to secondary follicle development, but inhibin α subunit was expressed at high levels independent of gonadotropin stimulation.

In PCO patients, α subunit mRNA was more abundant in thecal cells compared with the granulosa cell layer, contrary to normal ovaries, where α subunit mRNA is less intense in the thecal cell compared with the granulosa cell (25, 171). In addition, follistatin mRNA signal was present in the granulosa cells of normal small antral follicles but was not detected in the polycystic follicles (25). This would

suggest that PCO granulosa cells are exposed to more activin activity than normal granulosa cells.

In most ovarian cancer patients, elevated inhibin has been detected in the circulation and was suggested to be a potential marker for such cancers (172, 173) (also reviewed by Woodruff and Mather [4]). Among the six ovarian cell lines analyzed by Simone *et al.* (174), none expressed inhibin α subunit *in vitro.* However, four of these cell lines did express β subunits, and activin stimulated their cell growth in an autocrine fashion. These results were consistent with the observations in inhibin α–deficient mice, which develop ovarian tumors at puberty and have high circulating activin levels (29, 30). Crossing these animals with activin receptor (ActRII) gene–deleted animals resulted in animals that did not show the wasting associated with the α inhibin knock-out animals but that still developed gonadal tumors, suggesting that the development of tumors in the ovary is not mediated by signaling through the ActRII receptor (175).

Future Challenges

In spite of the truly impressive progress made over the last decade, understanding the physiology of inhibin and activin still presents us with challenges and unanswered questions. A system with multiple ligands, receptors, and binding proteins will inevitably be complex and difficult to understand. However, the very complexity of the many layers of regulation that have evolved around the inhibins is probably testimony to the important role that they play in development and physiology. Some of the most pressing unanswered questions concerning the roles of these factors in regulating reproductive function are outlined briefly below.

As discussed above, there is overwhelming evidence that an inhibin receptor which preferentially binds inhibin exists in the gonads. We await with interest the isolation, cloning, and characterization of such a receptor.

Similarly, *in situ* ligand binding studies show that some sites of activin binding in the ovary and testis cannot be competed by even a large excess of inhibin, while the same technique demonstrates cross-competition in the brain. Additionally, activin activity on Sertoli, but not germ cells, can be blocked by follistatin. Activin type II receptors have been shown to be present at both sites. Understanding the mechanism by which activin binding and activity can be differentially regulated in different cells remains a challenge.

Finally, the relative specificity of the different activins and inhibins for the different follistatins and receptors has yet to be determined. The exquisite control of the mRNA expression for these different subunits suggest that they must have different functions *in vivo.* This is backed by tantalizing hints *in vivo,* such as the differential regulation of inhibin A and inhibin B during the human menstrual cycle and the different activity of inhibin A and B on ovine and rat pituitary cell cultures. It seems almost impossible that we will not find that these hormones have different effects *in vivo.* We should continue to strive to find a way to put this knowledge to use in humans in diagnosing and treating reproductive dysfunction and disease.

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