MINIREVIEW

Inhibins, Activins, and Follistatins: The Saga Continues (44100)

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In 1991, I co-authored a minireview which appeared in this journal and dealt with two protein factors that had recently been isolated from ovarian follicular fluid (1). The two proteins, activin and follistatin, were discovered in the context of their effects on the reproductive system to stimulate and inhibit, respectively, the secretion of follicle-stimulating hormone (FSH) from the anterior pituitary gland (2–5). It was subsequently shown that activin, a member of the transforming growth factor–β (TGFβ) superfamily of growth/differentiation factors, had multifunctional roles in such diverse processes as morphogenesis, erythropoiesis, and reproduction, aside from its effects on FSH. Considering the co-localization of activin and follistatin in a wide assortment of tissues, including the gonads, pituitary, brain, and bone marrow, coupled with the fact that follistatin was a binding protein for activin led us to propose that these proteins functioned primarily at a local level to influence a number of different biological systems. It is safe to say some 5 years later that the preponderance of information published since 1991 has solidifed the concept that these cytokines are important local regulators of growth and differentiation in a number of diverse tissues at various stages of the life cycle. It is also likely that activin and follistatin function as classic endocrine modulators since they are present in the circulation. However, their role in this capacity has yet to be defined.

In the previous minireview, we chose not to elaborate on inhibin because there had been numerous review articles written on inhibin in the preceding years. However, recent findings that demonstrate a role for inhibin as a tumor-suppressing factor in the ovary (6) clearly identify inhibin as a legitimate local modulator, in addition to its well-defined role as a classic endocrine hormone. Although its precise role as a local modulator of reproductive function is still speculative, one could predict with some degree of certainty that inhibin's role as a local modulator of reproductive function involves its interaction with the activin-follistatin system.

The emphasis of this minireview will be on developments since 1991 involving inhibins, activins, and follistatins. While those developments are many, none is likely more significant than the identification of activin receptors. Other important developments concerning the physicochemical properties of these proteins have provided needed insight into the binding kinetics of the activin-follistatin complex, as well as the consequence of follistatin binding to membrane proteoglycans on the activin-regulating properties of follistatin. Another interesting discovery was the demonstration that inhibin, and to a lesser degree activin, bind to the protease inhibitor α_2-macroglobulin (α_2-M). Progress in assay development has allowed more accurate quantitation of these proteins in biological fluids. To interject some continuity with the previous review, I have provided updates regarding the importance of the activin-follistatin system to intrapituitary and intraovarian control of reproduction. The revelation that these proteins, particularly inhibin, are involved in tumorigenesis in the ovary was unexpected but underscored the diverse roles

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that these proteins play in biology. Finally, data are accumulating that suggest the contribution of these proteins to the etiology of certain disease states such as diabetes and atherosclerosis.

One aspect of activin biology that will not be covered in this review pertains to activin's role in early development. Indeed, it would seem that a large portion of work involving activin and/or follistatin published since 1991 has dealt with their role in morphogenesis. Because of the importance of this work to the field of developmental biology, and the abbreviated nature of the minireview, it is felt that a brief overview of this area would not be adequate, and the reader is referred to a review article by Kessler and Melton (7), which presents the current status and future directions for research dealing with the developmental effects of activin and follistatin.

Activin Receptors

The first reports of the presence of membrane surface receptors for activin were provided in the late 1980s. Binding of labeled activin was observed on granulosa cells (8), K562 human leukemia cells (9), and GH_3 pituitary sommatomammotrophs (10). Subsequent reports identified binding of both activin and inhibin to various cells and tissues including kidney (11), liver (11), brain (12), and germ cells (13). Based on these types of studies, a series of putative receptors was identified and called type I and type II receptors to be consistent with the nomenclature for TGFβ receptors (14). In 1991, Mathews and Vale (15) used an expression cloning approach in mammalian tissue culture cells to elucidate the structure of activin receptors. In this approach, cDNAs obtained from a library constructed from the murine AtT20 corticotroph cell line, an activin-responsive cell line, were transiently transfected into COS monkey kidney cells. Using emulsion autoradiography to visualize COS cells that bound [^{125}I]activin, a cDNA was obtained that encoded a type II receptor complex and was named *Act*RII (15). The cloning of this receptor led to the identification of *Act*RII complexes in other species (16–18), as well as to a second activin type II receptor subtype labeled *Act*RIIB (19–21). Interestingly, the activin type II receptors, similar to the TGFβ type II receptors, are constitutively active transmembrane serine-threonine kinases (22). Both *Act*RIIs bind activin A with high affinity, bind inhibin A with approximately 10- to 20-fold lower affinity, and do not bind TGFβ (22).

An activin type I receptor was cloned using polymerase chain reaction with oligonucleotides based on the *Act*RII sequence and appropriately labeled *Act*RI, although it is also called SKR1, Tsk7L, or ALK2, as well as other names (23–25). Unlike the type II receptors, *Act*RI does not bind activin, inhibin, or TGFβ. However, co-transfection experiments have demonstrated that expressed type I receptors can bind ligands in the presence of type II receptors (26, 27). Furthermore, type I receptor subtypes Tsk7L (28), ALK2 (29), and ALK5 (29) can each bind either TGFβ or activin in the presence of ligand-specific type II receptors. It appears that type II receptors allow recognition of the ligand by type I receptors, and association of the type I receptor with the type II receptor-ligand complex then initiates a complex signal transduction cascade resulting in an appropriate cell-specific biological response (30). Thus, type II receptors for the TGFβ family of growth/differentiation factors act as primary receptors exhibiting ligand specificity, while the indiscriminate nature of type I receptor binding to type II receptor-ligand complexes enables signaling for multiple receptor complexes that results in complex, but diverse, biological responses.

At the current time of writing, high-affinity receptors with selective specificity for inhibin have not been identified. As mentioned earlier, inhibin can weakly compete for binding of activin to activin type II receptors (22). In support of separate inhibin receptors, pharmacokinetic studies showed substantial uptake of radiolabeled inhibin, but not radiolabeled activin, in the adrenal gland 10, 60, and 1440 min after injection (11). However, such studies are fraught with caveats, including selection of time points that are not optimal for evaluating delivery of activin to the adrenal (11). In addition, this finding is in conflict with *in vitro* data demonstrating a direct effect of activin on primary fetal adrenal cell cultures (31). More recently, Xu and colleagues (32) concluded that most, if not all, biological activities of inhibin are likely mediated by binding to activin type II receptors, thereby preventing formation of the heteromeric complex of *Act*RII-activin-*Act*RI that is necessary for signal transduction. This conclusion was based on results showing that the heteromeric complex of *Act*RII-activin-*Act*RI is formed by binding of an activin β-chain to each receptor (32). Thus, while inhibin can bind to *Act*RII via its β subunit, the α-chain is not able to bind to *Act*RI, and the heteromeric complex critical for signal transduction is not formed. This theoretical assembly of a functional activin receptor complex is supported by data showing that the monomeric activin A is essentially devoid of biological activity, even though a single β subunit can bind to *Act*RIIB, albeit at 10% of the affinity of native dimeric activin A (33).

Binding of Inhibin and Activin to α_2-Macroglobulin

Previous work with hormone/growth factor–binding proteins demonstrated that these proteins could preclude accurate quantitation of the hormone/growth factor, particularly if the site or sites at which the binding

protein binds to the hormone/growth factor obscures epitopes recognized by neutralizing antibodies. In order to evaluate the binding characteristics of activin in serum, Schneyer and colleagues (34) chromatographed human serum to which they added radiolabeled activin. They found peaks of radioactivity corresponding to complexes with molecular weights of 60 and 230 kDa, as well as a peak corresponding to free activin. It was subsequently determined that the 60-kDa binding protein peak corresponded to activin complexed with follistatin, while the higher-molecular weight complex was determined to be activin complexed to α_2-M (35–37). Interestingly, α_2-M also bound inhibin and was found to be the major binding protein for both inhibin and activin in serum, while follistatin is the major binding protein present in follicular fluid (35). The binding of α_2-M to inhibin/activin is of low affinity ($K_d = 5 \times 10^{-7} M$), high capacity compared with the high affinity, low capacity binding characteristics of follistatin (37, 38). Finally, α_2-M did not alter the bioactivity or immunoreactivity of either inhibin or activin, unlike follistatin, which can neutralize the biological effects of activin (35).

Despite the demonstration of α_2-M binding to inhibin and activin, the physiological relevance of this binding relationship remains unclear. α_2-M is a tetrameric protein which was originally discovered as a serum protease inhibitor (39). Since then, however, α_2-M has been shown to bind a variety of cytokines/growth factors including TGFβ1 and 2 (see Ref. 40 for review). The available evidence suggests that the binding of α_2-M to cytokines/growth factors may facilitate interactions of these effectors with target cells and/or influence the clearance of the growth factor/cytokine. Indeed, recent evidence demonstrates that the conformation of the α_2-M molecule can influence the half-life of activin in circulation (38). Although the primary source of serum α_2-M is liver, local production of this molecule has been demonstrated in ovary (41), testis (42), and placenta (43). Taken together, α_2-M not only may facilitate transport of circulating activin and inhibin to their target tissue, but also may participate in the process by which these growth factors exert local actions.

Binding of Follistatin to Membrane Proteoglycans

Proteoglycans are glycosaminoglycans covalently linked to a core protein (44). One role of cell surface proteoglycans is to immobilize or sequester growth factors, thereby facilitating their actions at the cellular level (45, 46). It has been proposed that the importance of growth factor binding to membrane bound proteoglycans lies in the ability of the proteoglycan to reduce the dimensionality of ligand diffusion from three to two dimensions, thereby increasing the local concentration of the bound ligands at the plasma surface and the probability of their interaction with a high-affinity membrane receptor (47). In this regard, binding sites for follistatin on the surface of granulosa cells were found to be due to the binding of follistatin to heparan sulfate proteoglycans (48). It was subsequently determined that the heparan sulfate binding site of follistatin lies within a basic amino acid (aa)–rich region (aa 72–86) of the follistatin monomer (49).

The binding of follistatin to cell surface proteoglycans appears to underlie the ability of follistatin to regulate the biological actions of activin, particularly at the local level. For example, the greater potency of the 288–amino acid form of follistatin to suppress FSH secretion relative to larger follistatin isoforms appears to be attributed to a greater affinity of the shorter form for heparan sulfate chains of proteoglycans and not to differing affinities of the isoforms for activin (50). Likewise, exogenous heparin blocks the ability of follistatin to suppress FSH release from pituitary cells in culture, presumably by sequestering the follistatin and preventing its binding to cell surface proteoglycans (51). Consequently, diminished binding of follistatin to the proteoglycans reduces the amount of follistatin available locally to bind activin and neutralize its autocrine stimulatory effect on FSH. On the other hand, it has been reported that treatment of Xenopus ectodermal explants with heparinase, which removes glycosaminoglycan chains of proteoglycans, results in inhibition of mesodermal differentiation in response to signaling factors such as activin (52). These results would appear to indicate that follistatin, by virtue of its binding to proteoglycans, can enhance, as well as antagonize, the biological effects of activin. Clearly, the mechanisms that define the direction of follistatin's effects on activin bioactivity await a better understanding of the structural and functional relationships among follistatin, activin, activin receptors, and cell surface proteoglycans. Nonetheless, regardless of directional change, anchoring of follistatin to cell surfaces *via* binding to proteoglycans provides an effective and rapid means of buffering activin bioactivity.

Pituitary Production and Regulation

At the time of the first minireview, evidence was emerging that an intrapituitary mechanism regulating FSH existed, and that one of the players in this mechanism was activin B. This was based on two findings. The first was the demonstration that hypersecretion of FSH occurred following ovariectomy of hypophysectomized rats in which anterior pituitary tissue was grafted underneath the kidney capsule (1), and the second was the demonstration that co-culture of rat pituitary cells with a monoclonal antibody directed against recombinant human activin B dose-dependently inhibited FSH secre-

tion (53). Additional support for the existence of an intrinsic pituitary system governing FSH secretion that involved activin stemmed from studies in which administration of the same activin-B monocloncal antibody to hypophysectomized, pituitary-grafted rats reduced the FSH hypersecretory response to ovariectomy (54). Furthermore, the antibody partially suppressed circulating FSH levels during the estrous phase of periovulatory FSH secretion, thereby indicating that activin of pituitary and/or gonadal origin acting in an autocrine and/or endocrine fashion may be a physiological regulator of cyclic FSH secretion (54). More recently, Matzuk and colleagues (55) reported substantial reductions in serum and pituitary FSH levels in null mutant male and female mice lacking activin type II receptors. Finally, secretion of activin B dimer from rat pituitary tissue has been shown using immunoprecipitation of [^{35}S]-labeled cells followed by gel electrophoresis (56).

In order to gain greater insights into the importance of this level of regulation in controlling gonadotropin secretion, a number of studies have attempted to investigate the regulation of pituitary inhibin, activin, and follistatin biosynthesis and secretion, as well as activin receptor gene expression relative to changes in gonadotropin secretion. In this regard, removal of the gonads, which elicits increases in both luteinizing hormone (LH) and FSH secretion, resulted in increased expression of the inhibin/activin-β (57, 58), follistatin (59–62), and *Act*RI, *Act*RII, and *Act*RIIB genes (58, 62) in rat pituitary tissue. Interestingly, steroid replacement was effective in preventing gonadectomy-induced increases in inhibin/activin-β (57) and follistatin (60, 61) mRNA levels, but not in mRNA transcripts encoding the type II activin receptor (58). Further, administration of some gonadotropin-releasing hormone (GnRH) antagonists (61), but not others (59, 61), prevented increased follistatin gene expression following castration as did exogenous follistatin (59). Coupled with the findings that high-frequency GnRH pulses (61) and activin (59, 62) stimulate pituitary follistatin gene expression, and assuming that exogenous follistatin neutralizes endogenous activin bioactivity, it is likely that castration-induced increases in follistatin mRNA levels reflect responses to both GnRH and activin signaling.

Perhaps among the most significant findings regarding the regulation of gonadotropin synthesis and secretion by these factors were those that evolved from studies to examine the interactions of central GnRH and activin, inhibin, and follistatin. Foremost in this regard are experiments which demonstrate that activin can enhance not only pituitary FSH but also LH responses to GnRH *in vitro* (63). This is consistent with data showing that exogenous activin can stimulate secretion of both gonadotropins *in vivo* (64). One mechanism that might account for its effect on both gonadotropins is the stimulation of GnRH receptor synthesis by activin (65). On the other hand, the preferential effects of activin on FSH may relate to its ability to stabilize FSH-β mRNA transcripts (66). Like activin, inhibin also influences pituitary gonadotropin responses to GnRH. However, inhibin appears to exert its effect by blunting homologous stimulation of GnRH receptor synthesis, rather than by directly influencing GnRH receptor synthesis (67).

Analysis of pituitary expression of these factors during the rat estrous cycle has yielded some interesting data as well. For example, preovulatory surges of LH and FSH on proestrus and the secondary FSH rise on estrus are accompanied by increases in pituitary follistatin gene expression (59, 68). In contrast, no significant changes in inhibin/activin subunit or *Act*RII mRNA levels were observed thoughout the rat estrous cycle (68). Unfortunately, given the knowledge at hand one can only speculate as to a potential functional relationsip between increases in follistatin gene expression and cyclic gonadotropin release. In so doing, one must bear in mind that attempts to interpret changes in pituitary follistatin gene expression must consider the fact that the follistatin gene is expressed to some degree in all pituitary cell types, and that some of the changes in expression levels may be unrelated to reproductive events (69). In addition, one must consider the possible effects of high gonadotropin concentrations as seen during the preovulatory period on pituitary expression of follistatin and, for that matter, of inhibin and activin. Nonetheless, a scenario emerges that may prove useful in attempting to understand the complex relationships between central GnRH and intrapituitary activin and follistatin which eventuate in cyclic modalities of gonadotropin secretion in the rat (Fig. 1). According to this scenario, GnRH secreted during proestrous afternoon not only elicits gonadotropin surges, but also increases follistatin mRNA levels, which later are translated into follistatin protein (69a). Follistatin protein then may stimulate activin production late on proestrus and early estrus, since it has been shown that addition of follistatin to cultured pituitary cells stimulates secretion of immunoprecipitable dimeric activin B (56). The activin thus formed participates in evoking a secondary rise in FSH and might render a second increase in follistatin gene expression. Although the current scenario does not incorporate potential contributions by intrapituitary inhibin, support for a role of intrapituitary inhibin in regulating FSH secretion stems from studies showing that basal FSH secretion from cultured pituitary cells increases in the presence of an inhibin-α antibody (70).

While the aforementioned data were obtained using rodents or tissues derived from them, studies are now surfacing that demonstrate the potential of primate pituitary tissue to make activin, inhibin, and follistatin. In this regard, Attardi *et al.* (71) reported expression of the inhibin/activin βB and βA, but not the α, subunit genes in the monkey pituitary. In contrast to findings

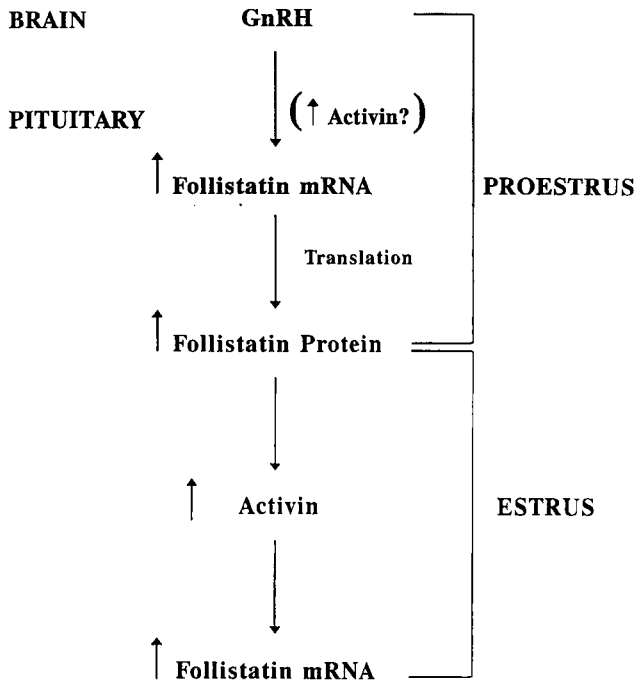

Figure 1. Hypothetical cascade of events in intrapituitary activin and follistatin expression during the periovulatory period of female rats which could contribute to cyclic gonadotropin release. See text for explanation and supporting literature.

in the rat, orchidectomy appeared to decrease βB gene expression (71). Interestingly, the full complement of inhibin/activin subunit genes as well as the *Act*RII gene is expressed in normal human pituitary tissue (72, 73). Thus far, demonstration of follistatin transcripts in primate tissues has not been reported, although measurements of serum follistatin concentrations in different reproductive conditions indicate that a significant contributor to serum follistatin in humans may be the pituitary gland (74). Clearly, future efforts are necessary to conclusively demonstrate production of these proteins in primate pituitary tissue, and, if so, whether or not this intrinsic system plays a role in neuroendocrine regulation of the menstrual cycle.

Intraovarian Production and Regulation

By 1991, it had already been established that activin and follistatin formed an important local regulatory system within the ovary that likely affected the dynamics of follicle selection. However, conflicting data existed as to the particular effects of activin in the ovary: *in vitro* studies supported a folliculogenic role for activin (enhancement of FSH stimulated estrogen, progesterone, and inhibin production, and induction of FSH receptors by LH), while a single *in vivo* investigation identified activin as a possible atretogenic factor (for review, see Ref. 1). Since the last minireview, reports have surfaced that appear to complicate this area further, particularly with regards to the *in vitro* effects of activin. For example, a number of investigators reported that activin inhibited progesterone secretion from granulosa cells obtained from the cow (75), monkey (76), and human (77, 78). Furthermore, similar to its effects on rat Leydig cells (79), activin suppressed androgen secretion from human thecal cells (80). On the contrary, an elegant study by Li *et al.* (81) demonstrated that addition of activin to monolayers of granulosa cells and oocytes obtained from 14-day-old, immature rats induced aggregation of granulosa cells around the oocyte, and a combination of activin and FSH resulted in the development of follicle-like structures. Moreover, activin was recently shown to stimulate DNA synthesis in rat granulosa cells *in vitro* (82).

Hillier and colleagues appear to have reconciled these divergent findings by proposing that granulosa cells display steroidogenic responses to activin that relate to the developmental state of the cell (76, 83). According to this scheme, activin enhances gonadotropin-stimulated increases in progesterone secretion from cells derived from immature follicles (i.e., primary), whereas activin attenuates gonadotropin-stimulated progesterone production from cells derived from mature follicles or those previously exposed to high gonadotropin levels that are likely to be in the process of spontaneous luteinization in culture. Indeed, activin has been shown to inhibit progesterone production from macaque luteal cells (84). On the other hand, activin enhances estrogen production induced by gonadotropins regardless of developmental state (83). Thus, activin appears to play a folliculogenic role in the early stages of follicle development, perhaps by being involved in the selection process, while in later stages activin might actually impede follicle growth and differentiation. If this were the case, one might be led to predict that follicles in the early stages of development either produce more activin or are better able to respond to activin relative to follicles in later developmental stages. To date, neither analysis has been performed. Once follicles are selected, activin production may wane as inhibin levels increase (85). Inhibin then may promote further development of the follicle to the preovulatory stage in view of its ability to increase thecal androgen production, thereby increasing the substrate for granulosa cell estrogen production (79).

While such an explanation does well to reconcile the *in vitro* data, it does little to explain the varied ovarian responses to activin administered *in vivo*. Not surprisingly, interpretation of *in vivo* effects of activin in pituitary-intact animals are complicated by the gonadotropin-releasing activity of activin. However, attempts to eliminate the contributions of pituitary secretions likewise have provided conflicting data of sorts in that intrabursal administration of activin to immature rats blocked the folliculogenic actions of pregnant mare's serum gonadotropins (PMSG) (86), while systemic ad-

ministration of activin to hypophysectomized, immature rats enhanced the ability of PMSG to stimulate increases in ovarian and uterine weights, and serum estradiol and inhibin levels (87). Hopefully, additional studies will be able to clarify what is likely to be complex actions and interactions of activin with other local regulators of ovarian follicular development. One must also bear in mind that the effects of activin are exerted not only on the somatic cells of the ovary but also on the oocyte, consistent with the identification of activin receptor subtypes on both somatic and germ cells (88, 89).

Progress has also been made in understanding the regulation of inhibin, activin, and follistatin production in the ovary. For instance, addition of FSH to primary cultures of rat granulosa cells stimulated follistatin gene expression (90–92) and production (91, 93). Similar to its effects on pituitary tissue, activin increased follistatin mRNA levels in granulosa cells (90). Miyanaga *et al.* (91), using ligand blotting technologies to semiquantitate secretion of inhibin/activin dimers and follistatin from rat granulosa cells, reported that FSH acting through the protein kinase A pathway favored inhibin secretion consistent with previous reports of FSH-stimulated increases in α subunit gene expression and immunoassayable inhibin secretion (for review, see Ref. 85). On the other hand, GnRH acting through the protein kinase C pathway stimulated activin production, while activation of both signaling pathways increased follistatin secretion. The observation that GnRH preferentially stimulates activin production, while FSH stimulates inhibin but blocks activin production, links activin once again with atretogenic processes since intraovarian actions of GnRH appear to promote follicle atresia (94). Also of considerable interest is the finding that GnRH stimulates follistatin gene expression in ovarian granulosa cells, which is reminiscent of its effects on pituitary tissue (61). In view of the ability of GnRH to stimulate ββ-dimer production from granulosa cells (91), and of activin to stimulate follistatin gene expression in both pituitary (59, 62) and granulosa cells (90), it is tempting to speculate that the effects of GnRH to stimulate follistatin in both the ovary and anterior pituitary gland are mediated by activin (Fig. 1).

Tumorigenesis

For many years, endocrinologists gained information as to specific physiological functions of endogenous hormones, growth factors, etc., by blocking their bioactivity *via* administration of antibodies or antagonists, or by inhibiting their synthesis (in the case of steroids and neurotransmitters) through administration of enzyme inhibitors. With the dawning of the molecular biology era in the mid-1980s, new approaches to assess the physiological significance of a hormone were developed. One such approach involved targeted deletion of cDNAs encoding particular proteins in question. These mutated gene constructs then were used to generate mice whose ability to produce the protein was "knocked out." This strategy was used by Matzuk and colleagues in an attempt to establish the importance of inhibin, activin, and follistatin to reproduction and development (95). In their initial studies, null mutant mice with a targeted deletion of the inhibition α subunit gene, which were therefore unable to produce inhibin, were generated. To their surprise, these inhibin knock-outs developed gonadal stromal tumors by 4–8 weeks of age (6). The appearance of these tumors at 4–8 weeks of age often were bilateral and multifocal. Spermatogenesis was initially normal but later became compromised, and serum concentrations of FSH were elevated as one might expect from the lack of inhibin negative feedback. Further manifestations of this gene deletion occurred after 2 months of age and included the appearance of a cachexic-like syndrome, liver necrosis, mucosal atrophy in the stomach, and reduced numbers of white and red blood cells and platelets (96). Death usually ensued by 3 months in females and 4 months in males. Interestingly, if these knock-out mice were gonadectomized before 6 weeks of age, no dramatic weight loss occurred, the liver and stomach histology were normal, and their survival increased into the eighth and ninth month of life. However, adrenal cortical tumors developed in the inhibin-deficient gonadectomized mice.

Another interesting discovery was the finding that inhibin knock-out mice exhibit elevated serum levels of activin A and B (96). Because inhibin can counteract the actions of activin in a number of tissues, the possibility arises that the increased serum activin levels could be causally linked to the development of gonadal tumors in inhibin-deficient mice. Along these lines, a role for activins as oncogenic agents has been obtained *in vitro* using cells from mice deficient in both the inhibin-α and p53 tumor suppressing genes (97). In that study, treatment of cells with follistatin or an antiserum to activin A inhibited tumor cell replication and blocked the stimulatory action of activin on cell growth. On the other hand, compound homozygous mutant mice deficient in both the α-inhibin subunit and *Act*RII still developed the gonadal tumors (98). These results, however, do not completely rule out a role for the activins in promoting gonadal tumorigenesis since activin signaling for this effect could be mediated by *Act*RIIB receptors or a yet to be identified activin receptor.

It is also possible that the elevated serum FSH levels observed in inhibin null mutant mice may elicit the development of gonadal stromal tumors. To test this hypothesis, Matzuk and colleagues generated inhibin- and GnRH-deficient mice by cross-breeding the GnRH-deficient hypogonadal mouse (hpg) with inhibin mutant mice (99). In contrast to inhibin-only null mutant mice, double knock-out mice failed to exhibit gonadal or adre-

nal tumors and survived for more than 1 year (99). However, histological analysis of ovaries taken from mice deficient in both inhibin and GnRH demonstrated premalignant or malignant phenotypes similar to the ovaries from inhibin null mutant mice. Interestingly, male mice deficient in inhibin and GnRH did not demonstrate any premalignant or malignant changes. These studies could be interpreted to suggest that the tumor-suppressing activity of inhibin may be indirect, being mediated instead by the high FSH levels, at least in the male. However, further work is necessary to establish more firmly the FSH hypersecretion as an etiology of gonadal tumorigenesis.

It is now known that tumor suppressors such as p53 oppose uncontrolled cell proliferation by at least two mechanisms: inhibition of proto-oncogene activity and induction of apoptosis (100). Accordingly, if inhibin exerted its tumor-suppressing activity by the same mechanisms, then it might be reasonable to assume that potential tumorigenic effects of activin may be attributed to prevention of apoptosis. However, activin has been shown to induce apoptosis in the liver (101) and, as was noted earlier, has been identified as an atretogenic factor in the ovary in some experimental paradigms. Clearly, further work is necessary to determine the involvement, or lack thereof, of activin in gonadal oncogenesis.

The identification of inhibin as a tumor-suppressing protein has bridged the reproductive field with yet another unrelated area, that of tumor biology. Indeed, serum inhibin is now proposed as a marker for ovarian cancer (102, 103) and, in particular, for sex cord stromal neoplasms (104, 105). Moreover, expression of inhibin/activin subunits and activin receptors have been reported in breast (106, 107) and prostate (108, 109) cancer cell lines. Interestingly, activin was found to inhibit the growth of MCF-7 breast cancer cells (107) and LNCaP prostatic adenocarcinoma cells (110, 111). At first glance, this would appear inconsistent with the supposition that activin could perhaps act as an inducer of some tumors. However, as was pointed out in discussing both intrapituitary and intraovarian control and actions of these proteins, activin, inhibin, and follistatin can regulate the expression of one another through complex autocrine/paracrine interactions. In this context, expression of the follistatin gene has been found in LNCaP cells (Dalkin A, personal communication).

Other Roles

In the previous minireview, the localization of activin and folistatin in diverse tissues predicted that activin and follistatin formed an intrinsic regulatory system in these tissues. As the number of tissues that expressed the inhibin, activin, follistatin, and activin receptor genes expanded, so did the number of studies to examine the functional significance of these proteins in those particular tissues and the potential role of these proteins in pathogenesis. For example, the demonstration of βA subunit mRNA in bone (112) and the structural relationship of the activins to the bone morphogenetic proteins prompted investigations on potential effects of activin on bone formation. These studies revealed that *in vivo* administration of activin A stimulated bone formation in either newborn (113) or young (5- to 6-week-old) rats (114), which was consistent with its *in vitro* mitogenic effects on the MC3T3-E1 murine osteoblastic cell line (115). Predictably, follistatin mRNA and protein were localized in bone tissue (116).

In the pancreas, activin stimulation of insulin secretion from rat pancreatis islets *in vitro,* prompted further studies to identify cell-specific expression of these proteins. Those results demonstrated βA subunit immunoreactivity in both A and D cells in one study (117), and A and B cells in another study (118). On the other hand, βB subunit and follistatin proteins were localized exclusively to pancreatic B cells (118). Interestingly, treatment of rats with streptozotocin, which destroys insulin-producing B cells, resulted in disappearance of follistatin and the βB subunits within 24 hr, prior to the disappearance of insulin (118). While these data demonstrate the existence of an intrinsic activin-follistatin system that may participate in the regulation of insulin, and perhaps glucagon and somatostatin, further work will be needed to assess the potential role of these proteins in the development of diabetes.

Another organ that appears to be a target tissue for activin is the liver. It was mentioned earlier that activin induced DNA fragmentation in hepatocytes indicative of apoptosis (101). Activin can also act on hepatocytes to stimulate glucose secretion (119). These results are consistent with the presence of *Act*RII mRNA in liver tissue (15). Indeed, these receptors appear to mediate the effects of elevated serum activin leads on hepatocellular necrosis in α-inhibin–deficient mice since inhibin knock-out mice deficient in *Act*RII do not develop liver pathology despite having elevated serum activin levels (98). Although cultured rat hepatocytes can synthesize and secrete follistatin (120), the existence of an intrinsic regulatory system involving activin and follistatin was questionable because neither the βA nor the βB subunit is expressed in the liver (1). Very recently, however, complementary DNAs encoding human βC (121) and Xenopus βD (122) subunits have been cloned. Using oligonucleotides based on the human βC sequence, Lau *et al.* (123) cloned the mouse activin βC cDNA and found it to be highly expressed in liver tissue. Therefore, it now appears that the liver harbors a novel intrinsic regulatory system which involves the production of a novel activin C dimer. A role for this system in controlling hepatocellular growth is

suggested by the finding that intraportal administration of follistatin to partially hepatectomized rats accelerates liver regeneration (124).

An effect of activin to modulate vascular smooth muscle was first reported in 1993 (125), followed shortly thereafter by the demonstration that vascular smooth muscle cells can produce both activin A and follistatin (126). Several reports then identified expression of activin and follistatin in arteriosclerotic lesions, which raises the question as to their importance in this pathological process (127, 128). More recently, Hubner *et al.* (129) reported that the activin A and B in skin may be important in wound healing since expression levels for the βA and βB subunit genes increased within the first week after wounding. Again, as one might predict, mRNA transcripts for both follistatin and activin receptors were localized in both normal and wounded skin (129).

Finally, the importance of inhibin, activin, and follistatin in regulating placental endocrine function is well documented and has recently been reviewed (130). Of particular interest, however, was the observation of increased circulating activin A levels during parturition (131). This increase occurred regardless of gestational age and was also accompanied by increased levels of serum follistatin (132). Although it is impossible at this juncture to determine whether parturition-associated increases in activin are a cause or a result of labor, it has been proposed that such increases in activin may facilitate oxytocin release (131).

Concluding Remarks

As mentioned in the opening to this minireview, many scientific advances have been made involving the inhibins, activins, and follistatins in the previous 5 years or so, which have contributed to a better appreciation for the pleotropic nature of these factors in biology. However, despite the major scientific advances and breakthroughs, our understanding of how these factors, particularly activin and follistatin, interact at the cellular level has progressed slowly. Granted, we have defined a series of important autocrine and paracrine ultrashort feedback loops that underpin the intrapituitary and intragonadal regulation of these factors. Nevertheless, a more thorough understanding of the interactions of activin, activin receptors, follistatin, and cell-surface proteoglycans is needed. For example, given that the affinity of follistatin binding to heparan-sulphate chains of proteoglycans is critical in determining the effectiveness by which follistatin modulates the actions of activin, it is critical that we gain a better understanding of the mechanisms that regulate proteoglycan dynamics inside the cell. While it is certain that further developments in the area of activin receptors will be forthcoming, an area that may not receive attention is the possible intracellular mechanisms underlying coordinate regulation of activin receptors and proteoglycan expression.

While other advances are inevitable, one must bear in mind that our understanding of the role of the inhibins, activins, and follistatins in various areas of biology is limited to the known factors at hand at any given time. This is best exemplified in the case of the liver, where we now are poised to make monumental advances in our understanding of liver growth and regeneration because of the recent identification of novel β subunits present in this tissue. Accordingly, the still somewhat murky nature of the intragonadal roles of these factors in controlling follicle growth and differentiation, and the uncertain importance of activin to embryogenesis may be due to as yet unidentified novel ligands and/or receptors belonging to this family of growth/differentiation factors.

One fact that many, if not all, scientists face is the necessity to go outside their fields of interest to acquire information that could impact on their own areas of research. Indeed, the ability to do this may be linked to how successful one is in today's highly competitive research environment. In reality, this necessity has existed from the beginnings of scientific investigation but is now acknowledged more than ever, due in part to rapid advances in both technology and communication. The nature of growth factor/cytokine research dictates this approach due to the ubiquitous distribution of these factors. Accordingly, those working in the inhibin and activin fields must be aware of work taking place in fields unrelated, at least in a systems sense, to their own. Thus, the next major advance in activin biology may come from opthalmology, as activin and follistatin are expressed in the retina, iris, and choroid layer (133, 134), or it may evolve from the discovery that serum inhibin A levels can be used to detect fetuses afflicted with Down's syndrome (135, 136). Wherever the discovery, researchers can then bring the information gleaned to bear on research questions posed in their respective fields of study.

In some respects, these last 5 years have been more exciting than the 5 years preceding the publication of the first minireview, during which the inhibins, activins, and follistatins were isolated and characterized. I would like to predict that the next 5 years and beyond will see an unprecedented explosion in the inhibin, activin, and follistatin field, not only from a basic science standpoint, but also—and, perhaps, even more so—from a clinical perspective. Wouldn't it be nice if everything could be so easy to predict?

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