MINIREVIEW

Activins, Inhibins, and Follistatins: From Endocrinology to Signaling. A Paradigm for the New Millennium

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It has been 70 years since the name inhibin was used to describe a gonadal factor that negatively regulated pituitary hormone secretion. The majority of this period was required to achieve purification and definitive characterization of inhibin, an event closely followed by identification and characterization of activin and follistatin (FS). In contrast, the last 15–20 years saw a virtual explosion of information regarding the biochemistry, physiology, and biosynthesis of these proteins, as well as identification of activin receptors, and a unique mechanism for FS action—the nearly irreversible binding and neutralization of activin. Many of these discoveries have been previously summarized; therefore, this review will cover the period from the mid 1990s to present, with particular emphasis on emerging themes and recent advances. As the field has matured, recent efforts have focused more on human studies, so the endocrinology of inhibin, activin, and FS in the human is summarized first. Another area receiving significant recent attention is local actions of activin and its regulation by both FS and inhibin. Because activin and FS are produced in many tissues, we chose to focus on a few particular examples with the most extensive experimental support, the pituitary and the developing follicle, although nonreproductive actions of activin and FS are also discussed. At the cellular level, it now seems that activin acts largely as an autocrine and/or paracrine growth factor, similar to other members of the transforming growh factor β superfamily. As we discuss in the next section, its actions are regulated extracellularly by both inhibin and FS. In the final section, intracellular mediators and modulators of activin signaling are reviewed in detail. Many of these are shared with other transforming growh factor β superfamily members as well as unrelated molecules, and in a number of cases, their physiological relevance to activin signal propagation remains to be elucidated. Nevertheless, taken together, recent findings suggest that it may be more appropriate to consider a new paradigm for inhibin, activin, and FS in which activin signaling is regulated extracellularly by both inhibin and FS whereas a number of intracellular proteins act to modulate cellular responses to these activin signals. It is therefore the balance between activin and all of its modulators, rather than the actions of any one component, that determines the final biological outcome. As technology and model systems become more sophisticated in the next few years, it should become possible to test this concept directly to more clearly define the role of activin, inhibin, and FS in reproductive physiology. Exp Biol Med 227:724–752, 2002

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The concept of a gonadal factor with an endocrine action at the pituitary is often traced to Mottram and Cramer (1). This activity was later named inhibin and defined as the water-soluble moiety derived from testes that could prevent the formation of castration cells in the pituitary (2). During the ensuing 30–35 years, the role of follicle-stimulating hormone (FSH) was defined and the activity of inhibin refined to selective control of FSH biosynthesis and secretion (3). With the purification of authentic inhibin protein (4, 5) and cloning of its α- and β-subunit genes (6–8), it was quickly realized that inhibins belonged to the emerging transforming growth factor β (TGFβ) superfamily of growth and differentiation factors, resulting in rapid proliferation of the entire field as a result of the high degree of structural and functional conservation within this family.

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During the inhibin purification effort, several fractions were found to stimulate FSH release from the pituitary, an observation that lead to the identification of activin as a homodimer of inhibin's β-subunits (9, 10). Another side fraction from the inhibin purification effort also exhibited FSH-release inhibiting activity, and upon purification and cloning, was discovered to be the distinct, monomeric protein follistatin (FS; Refs. 11, 12). The physiological significance of FS remained uncertain until its activin binding and neutralizing activity was identified (13, 14), thereby closing the loop and forever linking these proteins in a regulatory triangle.

Since the time of their identification and cloning, much has been learned about the biochemistry, molecular actions, signal transduction, and physiology of activin, inhibin, and FS, an effort greatly enhanced by discoveries in related TGFβ superfamily systems. The first reviews (15, 16) in this series provided scholarly and useful summaries of these defining events, including a detailed review of potential paracrine actions of these molecules. The second set of contributions (17, 18) contained expanded discussions of FS and its actions in regulating activin signaling and critically summarized the emerging intrapituitary regulatory system that controls FSH biosynthesis and secretion. The actions of activin and inhibin in a variety of physiological and pathophysiological settings such as cancer were also emphasized. Since that time, a great deal has been learned about the mechanism of activin action, including induction of gene transcription. More recently, advances have been gained in understanding mechanisms for the actions of FS, as well as the identification of potential inhibin coreceptors that can account for its potent endocrine activity. In addition, new findings on their effects on reproduction have been paralleled by expanded insights into actions of all three proteins in areas outside of reproduction, including development, neural induction, etc. Much of this work has been reviewed recently (19–21) and will only be briefly summarized here. Instead, we will concentrate on areas where recent advances belie emerging themes in the field and may set the stage for the next few years of research. There are also a number of relevant facets of this field for which existing reviews are still timely and complete, so we have tried to focus on specific areas where recent progress merits additional synthesis and compilation. For example, we have not included a discussion of the role of activin in the testis and refer interested readers to other reviews (18, 22).

The recent availability of sensitive and specific immunoassays has facilitated important advances that were derived largely from human subjects or tissues or primate models of human physiology. Thus, in the first section, we will summarize progress in inhibin, activin, and FS endocrinology by concentrating, wherever possible, on human studies. The second section will focus on autocrine and paracrine actions of activin and its regulation by FS and inhibin, concentrating on how these factors might interact to regulate activin actions in a physiological context. The next section will review recent advances in our understanding of the mechanisms whereby inhibin and FS act as extracellular regulators of activin action whereas the last section examines activin signal transduction and intracellular factors that modulate this pathway.

Endocrine Roles of Inhibin, Activin, and FS

Inhibin and Activin in Human Reproductive Endocrinology. *Inhibin and Activin Secretion and Endocrinology in the Female.* The development of assays specific for inhibin-A, inhibin-B, and activin-A advanced the understanding of inhibin and activin physiology in the human because all three hormones exhibit distinct patterns of secretion across the menstrual cycle (23–26). Inhibin-B increases across the luteal-follicular transition and reaches a peak in the midfollicular phase, whereas inhibin-A rises in the late follicular phase, reaching peaks in the midcycle and subsequently in the midluteal phase. Differential regulation of inhibin-A and inhibin-B biosynthesis partially accounts for their distinct patterns across the menstrual cycle (see below), whereas the unique secretion of the two inhibins also suggests distinct roles in FSH regulation and in follicular development, which remain to be elucidated.

Although the negative regulatory role of inhibin-A and inhibin-B on FSH has been established in pituitary culture (27), the negative regulatory role in the human and the relative role of inhibin-A, inhibin-B, and estradiol have yet to be firmly established. Repeat inhibin-A administration in the follicular (28) and luteal phases (29) suppressed FSH in the rhesus, but one dose of inhibin-A and inhibin-α antibodies failed to regulate FSH in the macaque (30). In addition, maintenance of luteal-phase estradiol levels alone suppressed the human luteal-follicular phase FSH increase (31). Taken together, these findings bring the negative regulatory role of inhibin-A, relative to that of gonadal steroids, into question in the primate.

Inhibin is not available for administration to humans; therefore, a number of studies have examined the negative feedback role of inhibin-A and -B indirectly in physiological situations in which there is dichotomous regulation of FSH and luteinizing hormone (LH). These studies hypothesize that increased inhibin selectively inhibits FSH. Inhibin-B decreases coincident, with the first detectable FSH rise during female reproductive aging (32–35), whereas estradiol is increased at this time (32). When studied longitudinally, it is apparent that inhibin-A also decreases (32). Inhibin-B decreases earlier than inhibin-A across the perimenopause coincident with a significant rise in FSH (36). These findings provide support for an important negative feedback role for inhibin-B and inhibin-A on FSH in the human, with FSH levels most responsive to early inhibin-B decreases.

Investigators have also examined inhibin levels in women with polycystic ovary syndrome (PCOS), in whom there is relative FSH suppression compared with LH. Serum inhibin α subunit, inhibin-A, and inhibin-B levels in PCOS

women were comparable with early follicular phase levels in normal cycling women (26, 37, 38) or were increased (39–41). Furthermore, inhibin subunit mRNA levels were lower in the granulosa cells of women with PCOS compared with normal granulosa cells from similarly sized follicles (42). These inconsistent findings suggest that elevated inhibin levels are not responsible for the relative FSH suppression in PCOS women.

The nearly irreversible binding kinetics of FS to activin and the neuralization of activin action by FS (43) suggest that the measurement of free activin and FS (see below) levels might be more meaningful to determine a potential endocrine role. Both free activin-A and total activin-A assays have been developed and used to examine activin changes across the menstrual cycle. However, there was no fluctuation in free activin-A levels across the menstrual cycle (44) and no difference in activin-B in the follicular and luteal phases (45). On the other hand, assays for total activin-A demonstrate a peak in the late luteal phase, before the onset of menses and around the midcycle (46), whereas there was no consistent change in follicular fluid activin-A during follicle maturation (47). Although the small increases in serum activin-A occur at times when FSH rises, other studies found no difference in activin-A levels in physiological situations with widely divergent FSH levels, such as in premenopausal compared with postmenopausal or castrate women (48, 49). Thus, activin-A is unlikely to play an endocrine role in the control of FSH secretion.

Regulation of Inhibin-A and Inhibin-B Secretion in the Female. The distinct patterns of inhibin-A and inhibin-B secretion in the human female suggest unique regulation of inhibin-A and inhibin-B by gonadotropins and stage of follicle development. These differences in regulation are apparent when comparing stimulation of secretion *in vivo* and *in vitro. In vivo,* FSH readily stimulates both inhibin-A and inhibin-B secretion in the early follicular phase, when small antral follicles are present (50, 51). In contrast, while both FSH and cAMP stimulate inhibin-A secretion from the granulosa cells of small antral follicles *in vitro,* neither stimulates inhibin-B (52). Furthermore, both FSH and LH stimulate inhibin-A from the preovulatory follicle but neither stimulate inhibin-B *in vivo* (51, 53). Consistent with the hypothesis that FSH stimulates inhibin-A but not inhibin-B, inhibin-A increases in follicular fluid with increasing follicle maturity whereas inhibin-B does not (47). The failure of FSH and cAMP to stimulate inhibin-B secretion from granulosa cells may be explained by the absence of a cAMP response element in the promoter of the inhibin-βB subunit (54, 55), in contrast with the promoters of the inhibin-α and βA subunits (55–57). In fact, several lines of evidence suggest that the βB subunit is regulated by activin, TGFβ, and bone morphogenetic protein-2 (BMP-2; Refs. 58–61) and that inhibin-B secretion requires insulin like growth factor-I (IGF-I) (52) and/or BMP-2 (61) and stimulation of the protein kinase C pathway (60). Taken together with the fact that a cohort of small antral follicles are growing in response to FSH in the early follicular phase, these observations suggest that increasing granulosa cell and follicle number likely account for the marked increase in circulating inhibin-B levels with the rise in FSH during the luteal-follicular transition. In contrast, inhibin-A levels increase in the late follicular and luteal phase both from increased granulosa cell number and direct FSH-stimulated inhibin-A secretion from individual granulosa cells.

Clinical Utility of Inhibin and Activin Measurements in Females. Inhibin-B and inhibin-A measurements in controlled experimental situations and in multiple clinical contexts have established their use as markers of follicle development. Preantral follicles secrete inhibin-B exclusively, whereas both inhibin-B and inhibin-A are secreted from small antral follicles, as evident from basal granulosa cell secretion (52) and their presence in follicular fluid (47, 62). Taken together with the differential regulation of inhibin-A and inhibin-B (51, 52, 60), inhibin-B levels across the luteal-follicular transition and follicular phase appear to reflect the total output of the growing cohort of small developing follicles. In contrast, inhibin-A levels in the early and midfollicular phases represent the sum of FSH- and LH-stimulated inhibin-A secretion from all antral follicles. In the late follicular phase, the rapid inhibin-A increase coincident with the marked increase in LH and demonstration that inhibin-A tracks with dominant follicle growth and demise (53) suggests that late follicular phase inhibin-A levels mainly reflect secretion from the dominant follicle.

In light of evidence for the use of inhibin-A and inhibin-B as markers of follicle development, the inhibins have been investigated as prognostic markers for women undergoing assisted reproductive technologies. It has been reported that day 3 inhibin-B levels <45 pg/ml predict a poor response to ovulation induction and decreased likelihood of achieving a pregnancy (63). Other studies suggest that there is great overlap in day 3 inhibin-B levels in women who become pregnant compared with those who do not and that no clear cutoff can be defined (64, 65). It has also been reported that inhibin-B levels are lower during a clomiphene citrate challenge test in women with diminished ovarian reserve (66) and correlate with ovarian response in gonadotropin releasing hormone (GnRH) agonist and FSH stimulation tests (38, 67, 68). However, other studies are not consistent with these findings (65) and again, there is a great deal of overlap between inhibin-B levels in normal and subnormal ovarian responses. There is some suggestion that inhibin-B levels during the early stages of FSH stimulation for ovulation induction predict the number of oocytes retrieved (69), and may be useful in monitoring ovarian stimulation for in vitro fertilization. Thus, inhibin-B as a predictor of success in assisted reproduction does not appear to have an advantage over FSH on day 3 or during a clomiphene challenge test, although inhibin-B may be useful as an early indicator of response during ovarian stimulation.

In addition to the ovary and testis, inhibin and activin subunit mRNAs have been identified in the fetus (70), pla-

centa (71), decidua (72), and fetal membranes (73). During a pregnancy cycle, inhibin-A rises 12 days after ovulation as a result of hCG stimulation of the placenta and corpus luteum (74, 75), peaks at approximately 42 days, then falls slightly thereafter (76). In contrast, inhibin-B remains low throughout pregnancy (76) despite βB subunit expression in the placenta and amnion. Although inhibin-A is produced by the corpus luteum, pregnancies using donor eggs (77) and frozen embryos (78) and studies during termination of pregnancy (79) suggest that the fetoplacental unit contributes the majority of inhibin-A during pregnancy. Inhibin-A, activin-A, and FS increase in the third trimester, with a dramatic increase in the final month, and disappear postpartum (46, 80–85).

The production of inhibin-A and activin-A during pregnancy has indicated that they might serve as markers of gestational diseases or fetal distress. Activin-A is elevated in preterm labor to the same extent as in normal labor, as well as in gestational diabetes (80), preeclampsia (along with inhibin-A; Ref. 86), and fetal growth retardation (87). However, further validation is needed before activin-A gains clinical use for these purposes. The 2-fold elevation of circulating inhibin-A in the second trimester in Down's syndrome pregnancies makes it an important and clinically useful test. Inhibin-A increases the Down's syndrome detection rate to 75% from 53% when added to alpha-fetoprotein, the β subunit of hCG, and maternal age as predictors (88, 93). Inhibin-A is lower in amniotic fluid of Down's syndrome pregnancies but needs further evaluation for early detection of Down's (94).

One of the most important developments in the field is the use of inhibin as an ovarian tumor marker (95). Inhibin α subunit, dimeric inhibin-A and B (96–99) and activin-A (100) have been detected in the serum of women with granulosa cell tumors and epithelial ovarian cancers. Inhibin assays directed to the inhibin α subunit are the most discriminating in detecting epithelial ovarian tumors, particularly mucinous epithelial tumors and granulosa cell tumors (96), whereas inhibin-B is also elevated in a majority of women with granulosa cell tumors (96, 97). The utility of inhibin α subunit assays has been confirmed for diagnosing granulosa cell tumors and evaluating tumor recurrence, with elevations preceding clinically detectable disease in some cases (101–103).

Inhibin Secretion and Endocrinology in Males. It is now clear that inhibin-B is the main inhibin secreted in the male (104, 105). Absence of inhibin-B after castration suggests that the testis is the primary source (105) and absence of inhibin-B in males with Sertoli cell only syndrome further localizes biosynthesis to the Sertoli cells (106). Although most studies in numerous species demonstrate inhibin α and βB subunit mRNA and protein in Sertoli cells, and to a small extent in Leydig cells (107–111), one study suggests that α subunit protein is found in Sertoli cells whereas βB is found in the germ cells (106), although it is difficult to determine how dimeric inhibin would form under these circumstances.

In addition to inhibin-B, activin-A and FS have been identified in seminal plasma, but only FS is present after vasectomy (112). Immunoreactive FS and activin βA were present in Leydig and Sertoli cells, and FS was demonstrable in epithelial cells of the seminal vesicle and prostate (112). Taken together, these findings suggest that activin-A is secreted by the testis, whereas the prostate makes a significant contribution to FS levels in seminal plasma.

A strong inverse relationship between FSH and inhibin-B in men across a continuum of spermatogenic activity provides evidence for a negative feedback role of inhibin-B on FSH in males (104, 113–116). As in females, FSH decreases selectively 2 days after inhibin-A infusion in nonhuman primates (117). Inhibin-B is expected to exert similar negative feedback regulation at the pituitary, although it has not been directly tested.

Regulation of Serum Inhibin-B Secretion in Males. As in females, serum measurements in males suggest that inhibin-B is regulated by FSH stimulation. Inhibin-B levels increase during the neonatal period and during puberty (118–121), times during which gonadotropin levels increase. GnRH replacement in GnRH-deficient men (114, 115) and FSH administration for acquired hypogonadotropic hypogonadism (122) increase inhibin-B levels. Supraphysiologic stimulation in normal males (105) and chronic FSH stimulation in oligospermic males (123) increase serum inhibin-B, whereas hCG does not (124). Despite these studies that suggest FSH stimulates inhibin-B, experimental evidence demonstrates that FSH does not stimulate Sertoli cell βB subunit expression *in vitro* (125–128) or *in vivo* (129). How can this paradox be resolved?

The regulation of inhibin-B secretion appears to be dependent on Sertoli cell proliferation, maintenance, and spermatogenesis in males, similar to the dependence of inhibin-B secretion on follicular growth in females. These processes require FSH, thereby providing the link between FSH and inhibin-B. For example, the increase in inhibin-B levels during the neonatal period and during puberty occurs at times in which Sertoli cell number increases (130, 131). FSH administration increases inhibin-B in prepubertal males in whom Sertoli cells can still divide (132). Induction of neonatal hypothyroidism to increase Sertoli cell numbers in rodents increased inhibin-B levels (133). Consistently, GnRH antagonist treatment and unilateral orchidectomy in nonhuman primates (120, 133, 134) decreased Sertoli cell numbers and inhibin-B levels. In males with GnRH deficiency, inhibin-B is detectable despite low FSH levels and correlates with testicular size (114, 115) and fertility potential (115), which are determined by the number of Sertoli cells. GnRH administration increased inhibin-B concomitant with testicular size, indicating increases in the Sertoli cell complement in these GnRH-deficient adults (115). Thus, the number of Sertoli cells partially determines inhibin-B levels.

In normal adult males, Sertoli cells no longer have the potential to divide (135), and inhibin-B increases only when spermatogenesis increases. In adult men with normal spermatogenesis, physiological FSH treatment does not stimulate inhibin-B secretion (124). In contrast, chronic FSH administration in men with hypospermatogenesis does increase inhibin-B along with sperm number (136). Inhibin-B levels increased after an initial drop in response to unilateral orchidectomy in conjunction with increased spermatogenesis in the remaining testis (134). In contrast, in men treated with androgens for contraception (105, 137), inhibin-B levels decreased consistent with the decrease in spermatogenesis (137). Therefore, spermatogenesis partially determines inhibin-B levels.

Experimental evidence is consistent with the hypothesis that spermatogenesis regulates inhibin-B levels. Inhibin secretion, as measured in assays directed at the inhibin-α subunit, is regulated by advanced spermatogenic precursors, particularly late spermatids (125, 138, 139). There is also evidence that dimeric inhibin-B levels are regulated by spermatogenic precursors. Inhibin-B levels are low in most subjects with Sertoli cells only syndrome (106, 140) and inhibin-B levels decrease with germ cell suppression using hormonal contraceptives (137, 141). In addition, one study correlated inhibin-B with spermatid number (140). Thus, either a product of spermatogenic precursors or the interaction with Sertoli cells appears to regulate inhibin-B. Taken together with the failure of FSH to stimulate inhibin βB mRNA, these findings suggest that number of Sertoli cells in the presence of normal spermatogenesis determines serum inhibin-B. FSH stimulation regulates inhibin-B in a permissive manner, through maintenance of the number of Sertoli cells, health, and ongoing spermatogenesis. At supraphysiological levels, FSH may stimulate inhibin-α subunit biosynthesis (124) with a resulting increase in inhibin-B formation. Therefore, regulation of inhibin-B production in males may be analogous to that in females; FSH doesn't directly stimulate inhibin-B protein production per cell but, rather, increases the number of cells (and follicles) making inhibin-B except when given at supraphysiologic levels.

Clinical Utility of Inhibin-B Measurements in Males. The potential regulation of inhibin-B levels by the interaction between Sertoli and spermatogenic cells suggests its use as a marker of spermatogenesis. Inhibin-B levels correlate with total sperm count (110, 113, 142, 143) and testicular volume (114, 115, 143). In men treated with chemotherapy for hematologic malignancy, inhibin-B decreased as expected based on known evidence for seminiferous tubule damage in these patients (144). The inverse correlation between inhibin-B and testicular biopsy score suggests that decreasing inhibin-B levels reflect progressive testicular damage (142). In addition, the potential to discriminate between spermatidic arrest in which inhibin-B levels are normal and Sertoli cell-only syndrome in which levels are generally low (106, 116, 140) point to a potential use in the evaluation of male infertility.

Despite these promising results using inhibin-B as a marker of spermatogenesis, it is unlikely to replace the testicular biopsy. Inhibin-B does not discriminate between spermatidic arrest and obstructive azoospermia in which levels are normal, nor can it distinguish between gradations of earlier spermatogenic arrest in which levels may be at the lower limit of normal (116). Finally, although inhibin-B levels are generally low, some patients with Sertoli cell-only syndrome have normal inhibin-B levels (106, 116, 140).

There remain two potential uses for inhibin-B in male infertility when used in conjunction with testicular biopsy. Patients with Sertoli cell-only syndrome and normal inhibin-B levels may have focal areas of spermatogenesis. Therefore, it is possible that additional testicular sampling in these patients could be useful to extract sperm. Second, inhibin-B predicted successful testicular sperm extraction in one study (145), although it did not predict outcome in an earlier study (116). Thus, further work will determine whether inhibin-B measurements in conjunction with a testicular biopsy will enhance male infertility treatment.

Follistatin: Is it an Endocrine Hormone? Because FS, like inhibin, was discovered in gonadal extracts as an inhibitor of FSH secretion from pituitary gonadotrophs, it was at first assumed that FS would also act in an endocrine manner. To test this hypothesis, development and validation of immunoassays was an essential step that has prompted substantial recent activity. The first such assay used an antiporcine FS antiserum that was raised against a mixture of FS isoforms (146). In the six in vitro fertilization (IVF) patients studied, stimulation with hMG and hCG to induce follicle growth resulted in a significant increase in serum FS, consistent with circulating FS coming, at least in part, from the ovarian follicle.

In contrast, a different heterologous FS radioimmunoassay (RIA), this one using an antibovine FS antibody, found serum FS concentrations of about 5 ng/ml, in both males and females, fertile or not, and regardless of menstrual cycle stage (147). These results suggested that gonadal FS production had no obvious influence on circulating FS levels and thus failed to support an endocrine role for circulating FS in regulating pituitary FSH release.

The first homologous FS RIA was developed with a mouse polyclonal antiserum to human recombinant FS288 (148). Neither activin-A nor inhibin-A had any effect on FS quantitation in this assay. FS concentrations of 8–10 ng/ml were detected in both males and females, with no difference observed across the menstrual cycle, nor were differences detected in normal women compared to post menopausal women or to women undergoing gonadotropin hyperstimulation for IVF. However, FS concentrations in follicular fluid were 3- to 175-fold higher than serum. Thus, although high FS concentrations are achieved in developing human follicles, the similarity in FS concentrations between nor-

mally cycling, gonadotropin-stimulated and postmenopausal women indicates that this follicular FS is not appearing in the circulation. Consistent with these results, serum and follicular FS were found to be biochemically distinct (149). These results support the concept that FS is compartmentalized with serum and follicles representing two distinct FS pools. Taken together with evidence that FS is produced in many tissues (see below), these assay results also suggest that FS is primarily an autocrine/paracrine modulator of activin action rather than a circulating endocrine hormone.

The next generation of assays used two antibodies to specifically recognize FS, thereby avoiding pitfalls with FS radioiodination. Interestingly, FS levels in serum at different stages of pregnancy increased up to 8-fold by week 36, suggesting a possible physiological role for circulating FS (85). This pregnancy-associated rise in FS was confirmed with different assays and a longitudinal study design, indicating that both activin-A and FS rise throughout pregnancy (81).

Two-site assays have also been developed to examine bioavailable, or free FS (150). Interestingly, free FS concentrations were undetectable in the same samples that were shown by RIA to contain 10–14 ng/ml of total FS. These results indicate that nearly all circulating FS is bound to activin. Other free FS assays have detected elevated FS levels in pregnant women, as well as in patients with chronic liver disease, chronic renal failure, advanced solid cancers, and hematological malignancies, suggesting that FS measurements might be useful as indicators of certain pathophysiological conditions (84).

Based on the ultrasensitive format successfully applied to development of inhibin and activin assays, Evans *et al.* (151) reported development of a two-site enzyme immunoassay for human FS288 that uses detergents to release activin from FS, thereby measuring total FS. Interestingly, FS315 cross-reacted about 10% in this assay. Again, FS levels in serum bore no relationship to menstrual cycle stage, averaging 0.62 ng/ml using recombinant FS288 as a standard. Given that these levels are 5%–10% of those found with the total FS RIA (148, 150), and this assay cross-reacts roughly 10% with FS315, these results are consistent with the earlier suggestion that circulating FS is FS315 whereas follicular FS is more like FS288 (149). This ultrasensitive assay was then used to examine serial samples from six normal pregnancies and confirmed the relationships of rising FS, activin-A, and inhibin-A with gestation. However, no difference in activin or FS levels were noted between women undergoing normal labor compared with caesarean deliveries, nor were changes detected during labor, suggesting that these proteins do not play an important role in human parturition (152). The rise of both FS and activin was also demonstrated in a larger cross-sectional/longitudinal study using a new FS RIA, although the absolute and relative levels of the two proteins differed considerably from earlier studies (153).

To test the hypothesis that circulating FS is indeed full-length FS315 (see below for more on FS isoforms), a new two-site immunoassay was developed with one antibody raised to the FS315 tail domain (154). This assay detected recombinant FS315, but not shorter forms, indicating that it was specific for the full-length molecule. When serum samples from normal men and women were examined, FS levels were comparable with the total FS RIA (148), confirming that the majority of FS in human serum is indeed FS315.

It is now clear from a number of FS assays using different formats and antibodies that FS does indeed circulate in the human and most likely in other mammals as well. Whether circulating FS is ultimately found to have physiological or pathophysiological significance remains to be determined. However, based on the data obtained so far, it is now evident that: 1. FS in the circulation is most likely full-length FS315 whereas FS in follicular fluid is predominately processed (see below), 2. Nearly all FS in circulation is bound to activin, 3. Ovarian FS does not contribute appreciably to circulating FS levels during follicular development, and 4. FS most likely acts as an autocrine/paracrine rather than endocrine factor in a number of tissues to regulate activin and other TGFβ superfamily members.

Local Regulation of Activin Action

From the beginning, inhibin was thought to be an endocrine feedback regulator of pituitary FSH release, and as detailed above—the evidence for this hypothesis is compelling. Whether inhibin has local actions within the gonad or in other tissues remains an active area of investigation and should now be addressable with the identification of candidate inhibin receptors (see below). Activin, on the other hand, although originally isolated from gonadal sources and also thought to have endocrine actions, now appears to be closer to its relatives in the TGFβ superfamily, which act primarily as local growth and differentiation factors in a wide variety of tissues. As detailed in earlier contributions to this series (16, 17), activin is synthesized in many adult tissues and cell types, and activin receptors have similarly been identified in those same tissues, a pattern more consistent with autocrine/paracrine mechanisms of action. In addition, circulating activin appears to be bound to FS virtually irreversibly (see below; Ref. 43), suggesting that for endocrine activin to have distant actions it would have to be released from FS by some proteolytic mechanism. Such a mechanism has been identified for the related BMPs, in which bioactive BMP is released by cleavage of its binding protein, chordin, by the metalloproteinase, tolloid (155–157). Until this type of mechanism is identified for activin, most attention has been, and will likely continue to be, focused on the local autocrine/paracrine actions of activin.

FS is synthesized in many tissues where activin is made, so that FS actions are also likely to be largely autocrine/paracrine in nature. However, substantial levels of FS circulate in blood, mostly bound to activin (see above), and

may act to facilitate clearance of activin and/or prevent diffusion of activin from its local site of action.

Earlier reviews in this series (17), as well as more recent contributions (21, 158, 159), have enumerated many possible actions of activin based on diverse approaches, including *in vitro* cell-based assays, assessment of activin and activin receptor biosynthesis, or gene inactivation paradigms. However, difficulties in establishing the physiological significance of these actions arise from the realization that although the activin or activin receptor knockout animals provided clues to potential activin actions *in vivo*, they have not yet provided definitive answers. In addition, because the FS knockout animals died within the first few hours after birth, assessment of the physiological roles of FS in the neonate and adult was not possible. Nevertheless, as discussed below, these seminal genetic studies provided a number of promising avenues upon which further research could be focused. It is also evident that the body of information in this field has become too extensive to review in its entirety. Thus, we will focus primarily on a relatively small group of activin actions for which a substantial body of evidence supports an important physiological role for activin in the adult and can thus serve as models for activin's actions elsewhere.

Regulation of Activin-Induced FSH Biosynthesis from Gonadotrophs. *The Role of Activin in Regulating FSH Biosynthesis.* Perhaps the most widely studied example of a potential physiological action of activin is regulation of FSH production in the anterior pituitary. Since the discovery of GnRH as the primary regulator of LH biosynthesis and release, there has been active investigation into a corresponding factor that could specifically elicit FSH release. The hypothesis that activin is the specific FSH-releasing agent is supported by three lines of evidence. First, hypophysectomized rats with pituitaries grafted under the kidney capsule still hypersecreted FSH when ovariectomized (17), indicating that an intact hypothalamic-pituitary connection was not required for FSH production. Second, monoclonal antibodies to activin-B could inhibit constitutive FSH release from plated pituitary cells as well as from hypophysectomized, pituitary grafted rats (17, 160). This observation is supported by the finding that deletion of the activin type II receptor leads to reduced FSH release in mice (161). Third, a number of investigators have demonstrated that inhibin/activin subunits, activin receptors, FS mRNA, and activin-B and FS protein are all made by pituitary gonadotrophs or folliculostellate cells (reviewed in Ref. 17), indicating that all necessary components for an autocrine/paracrine system of activin-mediated FSH biosynthesis exists within the pituitary.

At first, activin seemed to be a relatively modest inducer of FSHβ mRNA synthesis and FSH release (162), not much different from pulsatile GnRH (163). However, these studies were performed in plated pituitary cell cultures in which activin secreted by the pituitary cells themselves could accumulate (160, 164), thereby masking the true biological effects of exogenous activin. Using a perifusion system to remove endogenous pituitary activin, Weiss *et al.* (163) demonstrated that in the absence of endogenous activin, FSHβ biosynthesis became nearly undetectable. Moreover, continuous activin treatment upregulated FSHβ mRNA up to 55-fold compared with a 3-fold stimulation by pulsatile GnRH. This observation demonstrated that activin was far more important in regulating FSHβ mRNA production than previously appreciated, necessitating that both activin and GnRH be considered as potential physiological regulators of FSH biosynthesis and release.

With the development of the LβT2 gonadotroph cell line (165) and a luciferase-based reporter that contained 5.5 kb of FSHβ 5' regulatory sequence (166), it was now possible to verify these relationships in a relatively pure culture. Activin stimulated reporter activity approximately 4-fold whereas GnRH could account for a 2-fold stimulation (165). Because LβT2 cells synthesize endogenous activin-B (165), these results confirm the observations in pituitary cultures and emphasize the importance of activin as a regulator of FSH biosynthesis. Interestingly, activin also stimulated GnRH receptor expression in LβT2 cells (165) and αT3 cells (167), suggesting that activin may modulate gonadotropin biosynthesis in multiple ways. In addition, activin regulates biosynthesis of its own inhibitors (168), including matters against dpp (SMAD) (see below) and FS. Hence, the role of activin in the pituitary is manifested at many levels.

Regulation of Activin Activity. To be considered a physiological regulator of FSH biosynthesis and release, activin action had to be modulated because FSH levels fluctuate during the mammalian estrus or menstrual cycle, and these changes are critical for regulating follicular development. Although it was known that ovariectomy and subsequent removal of both steroidal and nonsteroidal feedback increased activin subunit mRNA synthesis (169, 170), it was not clear how local FS and endocrine-derived inhibin could interact with activin to regulate FSH release during the cycle. This problem was compounded by the observation that inhibins A and B circulated at levels 10-fold lower (25, 26) than that required for competition with activin binding to its type II receptor (171). The recent identification of candidate inhibin coreceptors (172, 173) that bind inhibin with high affinity provides one solution to this puzzle (see below).

In addition to this inhibin-mediated endocrine feedback regulation, components of an autocrine/paracrine system have emerged that include FS as an important regulator of activin-mediated FSH biosynthesis and release. Activin biosynthesis itself does not appear to be modulated to a significant degree during the rat estrous cycle (174); rather, it is levels of FS mRNA (174–176) and protein (177) that are modulated which, together with inhibin, can account for the critical secondary FSH rise. In fact, FS mRNA levels are directly regulated by GnRH pulse frequency, a parameter that varies across both estrous (178, 179) and menstrual

cycles (50, 180) and can differentially elicit release of LH or FSH (181). Thus, at faster frequencies, FS mRNA levels are increased, resulting in activin inhibition and reduced FSH production, whereas slower frequency GnRH pulses have the opposite action (175). Because it is known that GnRH frequency is slower during the secondary FSH rise in rats (178, 182) and at the luteal-follicular transition in humans (23), changing GnRH pulse frequency, acting via FS biosynthesis, could account for physiological patterns of FSH release in females. That this system can operate *in vivo* was demonstrated by administration of FS288 to rats via cannulation of the carotid artery, a treatment that reduced FSH release (183).

So where does inhibin fit into this scenario? As detailed above, we now know that inhibin biosynthesis is regulated during the estrus and menstrual cycles such that inhibin-B peaks early in the follicular phase while inhibin-A peaks in the mid luteal phase. It is therefore likely that rising inhibin-B levels in the midfollicular phase act at the pituitary gonadotroph to offset activin signaling and suppress FSH biosynthesis from early follicular phase levels. Similarly, elevated inhibin-A levels in the luteal phase likely act to suppress FSH, and the fall in inhibin-A at the end of the luteal phase creates an environment whereby FSH levels can again increase. As mentioned above, the slowing GnRH pulse frequency in the luteal-follicular transition (50) leads to decreased FS levels, so that the combined effect of reduced inhibin-A and FS concentrations lead to greater activin activity and FSH release. Support for this model at the cellular level comes from recent studies showing that both FS and inhibin can decrease FSHβ mRNA levels in gonadotrophs and gonadotroph cell lines through specific effects on transcription (165, 166, 184). However, the precise mechanism(s) for this regulation are not entirely clear because sex steroids regulated both FS and activin subunit mRNA levels (185), activin stimulated FS mRNA (186), GnRH blockade reduced FS mRNA levels (187), and passive immunization of inhibin resulted in increased FS mRNA (187), suggesting that the interaction of all of these factors, both endocrine and autocrine, determines the level of FSHβ mRNA biosynthesis and FSH release. Thus, one emerging theme is that both inhibin and FS can act coordinately as extracellular regulators of activin action to regulate the timing, duration, and amplitude of FSH secretion.

Recent studies have added a new wrinkle to this seemingly complete picture. It now appears that both BMP-6 and -7 can modulate FSHβ biosynthesis in gonadotrophs and gonadotroph cell lines (188) and that basal FSH biosynthesis can be inhibited by antibodies to BMP-7. Moreover, both BMPs are produced in the anterior pituitary and LβT2 gonadotroph cell lines, suggesting the presence of another system for regulating FSHβ biosynthesis. Because in sufficient concentrations FS can bind and neutralize both BMPs and BMP-7 can bind to ActRII, it is presently difficult to differentiate between activin-mediated and BMP-mediated FSH biosynthesis to assess the relative physiological relevance of the BMP system. Perhaps there are redundant regulatory systems for FSH biosynthesis, or alternatively, perhaps both activin and BMPs drive FSHβ mRNA production and the same set of regulatory molecules act on both growth factors to modulate FSH production. No doubt this is fertile ground for more detailed analysis.

Regulation of Ovarian Follicular Development. Although inhibin clearly has endocrine actions at the pituitary, the purification of inhibin, activin, and FS from gonadal sources suggests that all three also have biological actions within the gonad. Although potential actions of these molecules in the testis remain speculative, support for local actions in the ovary is more substantial. Provocative experiments by Woodruff *et al.* (189) showed that direct injection of inhibin and activin under the rat ovarian bursa could drastically affect follicle development, with inhibin and activin acting in apparent opposition. Further, *in vitro* studies demonstrated that activin could regulate granulosa cell proliferation, FSH receptor number, FSH-mediated gene expression (190), and steroid biosynthesis (reviewed in Refs. 15, 17, 191). FS was subsequently demonstrated to bind to granulosa cell-surface proteoglycans, where it could sequester activin (192) or form a barrier to exogenous activin (193), thereby inhibiting its actions. One synthesis of these observations suggested that the action of activin on naive, immature granulosa cells is to promote proliferation or differentiation, as detected by increase in granulosa cell number, FSH-responsiveness, and inhibin and estrogen production, whereas in mature granulosa cells, activin seems to retard or prevent luteinization (191). This is supported by the observation that activin could inhibit progesterone production from human-luteinized granulosa cells, an effect that could be inhibited by FS (194). The actions of inhibin also extended beyond granulosa cells, where it was observed that treatment of theca cells with inhibin could enhance LH-mediated androgen production. This led to the hypothesis that granulosa cell inhibin might diffuse to the thecal layer, where it would enhance production of the androgen precursor that, upon returning to the granulosa cell, would be converted to estrogen by aromatase (195).

Earlier studies on inhibin, activin, and FS gene expression and protein production were also informative in suggesting possible actions for these proteins within the ovary. In the monkey and human, pre-antral and small antral follicles expressed mostly βB subunit mRNA. As follicles increased in size, they contained more α- and βA subunit and FS mRNA whereas βB transcript levels seemed to remain relatively constant (196, 197). Overall then, it appeared that activin-B and, later, inhibin-B might be produced in small developing follicles, which is replaced by inhibin-A and activin-A and neutralized by FS as follicles approach the pre-ovulatory stage. This scenario fit with the proposed actions of these hormones based on *in vitro* studies enumerated above. In addition, synthesis of inhibin would divert some β-subunit protein away from activin production, fur-

ther reducing the influence of activin, and increased FS would work toward inhibiting activin as follicles mature.

This concept was recently tested in humans by aspirating follicles from normal women at different stages of the menstrual cycle after daily serum sampling through one complete observation cycle to document normal follicle development (198). Measurement of inhibins A and B, activin-A, FS, and steroid hormones demonstrated that, like estrogen and progesterone, biosynthesis of inhibin-A increased significantly with follicle size and maturity whereas inhibin-B levels did not correlate with either size or maturity. The relationships of FS and activin were more complex and variable but in follicles larger than 10 mm, FS appeared to increase with follicle size. However, in almost all follicles, free-FS was always in excess of activin, suggesting that in follicular fluid, all activin is bioneutralized, consistent with the concept of activin activity being suppressed as follicles develop. These relationships were verified at the mRNA level in a subset of follicles for which sufficient mRNA was available (42). Using quantitative reverse-transcription polymerase chain reaction, it was shown that α- and βA subunit mRNA increased with follicle size and maturity whereas βB mRNA did not correlate with either parameter. Interestingly, FS mRNA levels did not change significantly during follicle development, nor did the ratio of FS315:FS288 alternate transcripts, with this ratio being approximately 3:1 at all stages. These results in carefully characterized normal women are consistent with the notion developed from animal and human anatomical studies (62, 196, 197, 199) that conversion from a largely activin dominant to an inhibin-A/FS--dominant microenvironment is important for normal antral follicle development.

To test this concept further, results from normal women were compared to women with PCOS, where follicles are arrested at the 5–8 mm stage. When compared with size-matched normal follicles, both inhibin α- and βA subunit mRNA levels were significantly lower (42), which is consistent with a preliminary observation that inhibin-A protein is also significantly reduced in follicular fluid aspirated from these PCOS follicles compared with size-matched normal follicles (200). Taken together, these observations suggest that reduced inhibin biosynthesis is associated with follicular developmental arrest in women.

Further support for this concept can be found from a variety of genetic approaches that result either in disruption of follicle development or the inhibin/activin/FS system itself. For example, deletion of the gonad-specific transcription factor associated protein $TAF_{11}105$/Taf4b resulted in follicular developmental arrest in homozygotes, a finding that correlated with expression of $TAF_{11}105$/Taf4b in normal granulosa cells. Comparing gene expression between homozygotes and heterozygotes for thousands of ovarian genes using gene expression profiling and RNase protection assays, the most severely downregulated genes included inhibin/activin α, βA, and βB subunits as well as FS and aromatase (201). Moreover, deletion of the inhibin α-subunit resulted in loss of fertility along with the development of aggressive stromal tumors in virtually all animals, which prevented assessment of the true developmental potential of these follicles (202). Because disruption of the activin βA (*Inhba*) and FS (*Fst*) genes caused perinatal lethality, the effect on follicle development could not be directly assessed, but deletion of activin βB (*Inhbb*) did not disrupt follicle development, although fertility was reduced because of a deficit in nursing their offspring (203). In a clever follow-up experiment, Brown *et al.* (204) replaced the activin-βA coding sequence with the sequence for mature activin-βB linked to the promoter and pro region of the βA subunit. This manipulation rescued the craniofacial phenotypes, which caused perinatal death in the βA knockouts but did not restore normal female fertility or follicle development in adult females, indicating that the βA subunit, either as inhibin-A, activin-A, or both, is necessary for normal follicle maturation. Finally, overexpression strategies, including FS (205) and inhibin α-subunit (206, 207) resulted in disruption of normal follicular development and fertility, indicating that dosage of these proteins, as well as perhaps timing of expression, is critical for maintenance of normal fertility.

In vitro culture of ovarian follicles has permitted examination of the actions of activin in regulating follicle maturation. As detailed in earlier reviews, the demonstration by Li *et al.* (208) that treatment of immature rat granulosa cell/oocyte cocultures with FSH and activin resulted in formation of apparently intact follicle structures clearly demonstrates the power of activin signaling on ovarian cells. Using culture of secondary follicles dissected from 14-day-old mouse ovaries, Smitz *et al.* (209) found that activin secretion by these growing follicles increased 20- to 50-fold over the 12-day culture period during which the follicles achieved pre-ovulatory status. Moreover, addition of activin to the FSH-supplemented medium dramatically increased follicle volume. Growing follicles produced both estradiol and inhibin, and this hormone secretion was increased by activin. These actions of activin could be blocked by addition of excess FS. Activin treatment also augmented growth-promoting effects of growth hormone (GH) and IGF-1 in pre-antral follicles from immature mice (210). Interestingly, the actions of activin were not identical on pre-antral follicles from mature or immature females. Follicles from immature mice increased in size in response to activin, or activin plus FSH, but not to FSH alone, whereas pre-antral follicles from adult females grew in response to FSH alone, a response inhibited by activin cotreatment (211).

When comparing activin effects to those of TGFβ in adult versus immature pre-antral follicles, TGFβ appeared to promote growth of adult follicles, including increased estradiol and inhibin production, but had no effect on immature follicles, whereas activin treatment had the opposite effect (212). Activin from secondary follicles may even inhibit growth of smaller follicles (213). Collectively, these studies demonstrate that activin has a variety of growth-

promoting actions in immature follicles consistent with findings from granulosa cell culture studies (191). Moreover, they demonstrate the value of *in vitro* follicle culture for dissecting actions of activin and related growth factors but also emphasize the challenge in determining the physiologically relevant actions of activin *in vivo* from these approaches alone. Nevertheless, it is clear that activin has important actions in regulating development of ovarian follicles.

It is now increasingly evident that factors elaborated by oocytes, including members of the TGFβ superfamily, have a profound influence on follicle development (214, 215). Moreover, activin treatment stimulated meiotic maturation in oocytes ranging from zebra fish (216) to rodents (217, 218) to humans (219), and it now appears that even the epidermal growth factor (EGF)-enhanced meiotic maturation in zebra fish oocytes is mediated, at least in part, by activin (220). Importantly, recent studies demonstrate that human and mouse oocytes possess all activin receptor subtypes but do not synthesize inhibin/activin α, βA or βB subunit mRNA, or presumably protein (221). This finding raises the possibility that the actions of activin within the follicle result not only from autocrine actions on granulosa cells but could also derive from paracrine actions of granulosa cell-derived activin on oocytes. Therefore, activin could merit inclusion in the growing list of intrafollicular communication pathways involving TGFβ superfamily members, which currently includes GDF-9 and BMP-15.

Nonreproductive Actions of Activin. Over the last few years, a number of nonreproductive actions of activin have been identified and characterized. The following section is intended to provide a brief summary of areas under active investigation that should produce important milestones in the next few years.

Wound Repair. Both activin βA and βB subunit mRNAs and protein are expressed in normal adult mouse skin (222, 223), whereas FS mRNA and protein are made by fibroblasts from many tissues, including skin (224). Interestingly, FS knockout mice have defective whisker development and shiny, taut skin (225) whereas activin βA knockout mice lack whiskers and have abnormal whisker follicles (226). Activin expression, on the other hand, was induced by injury (227), as well as by TGFβ, EGF, and PDGF, all of which are released from platelets upon hemorrhage. Interestingly, activin induced the proliferation of keratinocytes (223). These observations collectively suggest a role for activin in wound repair.

This hypothesis was examined by creation of transgenic mice expressing activin βA subunit under the direction of the keratin 14 promoter (222). Transgenic mice were both viable and fertile but were smaller than wild-type litter mates. Homozygote mice had thickening of both the dermal and epidermal layers, and the fatty tissue was replaced by connective tissue. Wounds in these mice demonstrated increased granulation tissue, higher cell density, and enhanced connective tissue deposition, all of which suggest that activin expression was enhancing the process of wound healing.

Kidney Tubule Morphogenesis. Activin subunits, activin receptors, and FS have all been identified in the kidney. In addition, and transgenic mice overexpressing a dominant negative type II activin receptor under control of the β-actin promoter were infertile and had, among other defects, a hypertrophic kidney cortex with 1.8-fold more glomeruli (228). This was consistent with the earlier finding that activin inhibited branching morphogenesis of the ureteric bud during kidney development (229). To look at molecular mechanisms that underlie this action, MDCK cells, derived from collecting ducts, were examined and found to form branching tubules in response to hepatocyte growth factor (HGF; Refs. 230, 231). Activin-A inhibited this morphogenesis when added simultaneously with HGF (232). Interestingly, FS not only blocked the activin effect, but could also induce morphogenesis in the absence of activin or HGF. This action was mimicked by a dominant-negative activin receptor. Taken together, these results suggest that any actions that block endogenous activin-A enhance branching morphogenesis. Furthermore, HGF suppresses production of activin subunit biosynthesis, suggesting that HGF may be acting by suppressing the inhibitory effects of activin (233).

Does activin or FS have any role during regeneration after ischemic injury? In normal rats, βA subunit expression was markedly increased upon ischemic injury. On the other hand, FS was abundantly expressed in normal rat kidney tubules, and its expression is reduced by injury (233). Infusion of FS immediately after arterial blockage reduced the number of histological defects and apoptotic tubular cells while leading to increased tubular proliferation, suggesting that FS administration reduced tissue damage and promoted repair processes. Administration of activin-A, on the other hand, lead to reduced DNA synthesis and increased apoptosis in many cell types (234). Thus, the balance of activin and FS may be critical for regulating regenerative processes in kidneys after ischemic injury.

Systemic Inflammation. Although the role of TGFβ in inflammation has been known for some time (235), the role of activin may also be important, albeit less well defined. Activin was purified as an erythroid differentiation factor (236), and it induces hemoglobin accumulation in erythroleukemic cells (237), indicating that activin stimulates differentiation of cells in this lineage. In fact, there are a growing number of responses that are quite different for TGFβ versus activin-A, suggesting that both of these molecules may have physiological roles in systemic inflammation (238). In this regard, use of the sheep model has been particularly helpful, showing that surgical procedures, known to activate the acute phase reaction, led to release of large amounts of FS (239). FS was also released in response to a lipopolysaccharide (LPS) or interleukin (IL)-1β challenge (239). Activin, on the other hand, is increased within 30 min of an LPS challenge and is biphasic with a second

peak at about 4 hr, returning to pretreatment levels by 5 hr, which is much earlier than seen for FS levels under the same treatment. The activin-A profile was quite similar to that of tumor necrosis factor-α (238). Thus, this inflammatory response is atypical in that serum activin and FS levels change independently. Moreover, the rapid, robust response of activin secretion is fascinating because activin is not typically stored in cells, which raises an interesting issue about its source. These questions notwithstanding, current evidence points to a role for activin and FS in mediating at least some aspects of the response to inflammation and possibly infection.

Bone Metabolism. Both TGFβ itself and the TGFβ superfamily subgroup of BMPs both have well-documented actions regulating bone resorption and/or formation. Activin was purified from demineralized bone, is produced in embryonic bony tissue, and stimulates proliferation and collagen synthesis of osteoblastic cells (see Ref. 240 for review). Bone cells from rat calvaria and osteoblastic cell lines also secrete bioactive activin (241). During endochondral bone formation, activin:FS ratios fluctuate in correlation with chondrocyte maturation and bone cell development (242). Moreover, activin was detected around fractures in proliferating and osteoblastic cells at higher levels than in normal bone, suggesting that activin is involved with new bone formation. Local injection of activin around fractures facilitated the repair process and strengthened the healing bone (243, 244). Taken together with the identification of activin receptors on osteoblasts, these observations suggest that activin, and its regulation by FS, has a role in bone repair processes (245). Additional regulation of activin action could result from changes in the balance between locally produced activin and inhibin from endocrine sources, which together could regulate remodeling in adult bone. For example, reduction in circulating inhibin associated with menopause has been postulated to increase osteoclast activity and, potentially, osteopenia (246).

Within the bone marrow compartment, activin has well-known effects on erythroid progenitor differentiation (236). Bone marrow-derived stromal fibroblasts are the primary source of both activin and FS in the marrow. In addition, activin-A production is enhanced by IL-1β and LPS, whereas FS production is inhibited by this treatment. In contrast, interferon-γ stimulated FS secretion and inhibits activin production. Thus, in this context, inflammatory cytokines regulate activin production and presumably action via reciprocal actions of IL-1β versus interferon-γ (247).

Extracellular Modulators of Activin Action: Inhibin and FS

As suggested above, many lines of inquiry indicate a role for FS and inhibin as extracellular modulators of an often-constitutive autocrine/paracrine activin stimulus through distinct mechanisms. Although the ability of FS to regulate activin depends on its concurrent synthesis and cell surface binding, the ability of inhibin depends on the presence and function of receptors for circulating inhibin. Acting alone or together, these two extracellular proteins modulate activin signaling before activin binds its receptor (FS), or before signal transduction is fully activated (inhibin), thereby providing the first of several levels for regulating activin action. In fact, the presence of so many mechanisms for regulation of activin action (see Ref. 248 and below) argues persuasively for its importance to normal physiology and potential for initiating pathophysiological outcomes when not properly controlled.

Mechanism(s) of Inhibin Action: Inhibin Receptors. As detailed above, it is now clear that inhibin is an important feedback regulator of FSH secretion in human males and females, as well as in other species (27). Yet, until recently, the only known mechanism whereby inhibin might influence activin-mediated FSH production was via its ability to compete with activin for binding to its type II receptor (171), an action that would require inhibin concentrations to exceed those of activin by more than is currently achieved during normal menstrual/estrus cycles (26, 249). Inhibin was also quite potent in many *in vitro* assays, again at levels inconsistent with the competition mechanism. These observations led to the arduous search for an "inhibin receptor," the existence of which was supported by *in situ* binding studies in which gonadal inhibin-specific binding sites were identified (250). The first specific inhibin-binding moieties, isolated by affinity chromatography from inhibin α-subunit knockout mouse gonadal tumors (251), ranged in size from 72–130 kd and bound iodinated inhibin, but not activin.

Applying this same strategy to bovine pituitaries, a richer and more abundant source of inhibin binding proteins, Chong *et al.* (252) purified a novel inhibin-binding protein termed p120/InhBP. The p120/InhBP sequence matched a large membrane-bound proteoglycan in the immunoglobulin/cell adhesion superfamily. Recent evidence suggests that inhibin induces association between p120/InhBP and ActR1B (ALK4), thereby preventing formation of functional activin-ActRII-ActRI complexes and disrupting activin signal transduction (253). Given that this inhibin-binding protein is expressed in pituitary gonadotrophs, as well as in the gonads, it represents a candidate inhibin receptor that could account for a majority of inhibin's actions.

Another long-term search for the elusive inhibin receptor identified the previously characterized TGFβ Type III receptor, betaglycan, as a specific inhibin binding protein (172). High-affinity inhibin binding sites (Kd = 0.2 nM) were detected in 293 cells transfected with betaglycan and ActRII cDNAs whereas without the betaglycan, inhibin-binding affinity was measured at 6.3 nM, consistent with binding to ActRII alone. Affinity cross-linking experiments demonstrated specific inhibin binding to betaglycan and, furthermore, that inhibin and betaglycan formed complexes with ActRII, but not ActRI, suggesting that inhibin binding to betaglycan disrupts activin signaling by sequestering

ActRII into nonfunctional complexes. Because activin could not compete with inhibin for binding to betaglycan, it is thought that this association is mediated via the α-subunit of inhibin. Inhibin-binding sites have been further localized to the cytoplasmic portion of the betaglycan ectodomain, and could be competed by both inhibin and TGFβ2 (254). The biologic activity of betaglycan in the inhibin-signaling pathway was shown by transfection of inhibin-insensitive corticotrope cells with betaglycan, rendering them inhibin sensitive (172). Betaglycan was also localized to many of the previously identified inhibin sensitive tissues (172). Taken together with the p120/InhBP results, these studies indicate that there at least two specific pathways whereby inhibin can oppose activin signaling, although their physiological significance remains unproven because neither has been yet been demonstrated to modulate a physiological inhibin action such as regulation of FSHβ biosynthesis by gonadotrophs.

In fact, there may be additional inhibin binding proteins or co-receptors. Harrison *et al.* (255) characterized binding of ^{125}I-inhibin to mouse Sertoli and Leydig cell lines, demonstrating both high- and low-affinity binding sites. Using cross-linking studies, the high-affinity component of inhibin binding was found to consist of at least five proteins ranging in size from 70 kd to greater than 200 kd. Three of these protein complexes could be immunoprecipitated with antibodies to betaglycan but none with P120/InhBP antiserum, suggesting that several proteins, in addition to betaglycan and P120/InhBP, may mediate inhibin binding and signal transduction in these cell lines. Given the widespread expression of betaglycan, the expression of p120/InhBP in reproductive tissues, and the presence of additional inhibin binding components, it is clear that the elucidation of the signaling pathway of inhibin is a major challenge for the next few years.

Mechanism(s) of FS Action: The FS-Related Gene Family. *Follistatin Structure and Activity.* Bioactive peptides and proteins use a number of mechanisms to maintain their activity at an appropriate level. These may include transcriptional and translational control, processing from larger biosynthetic precursors, responses to secretory signals, association with transport proteins, exposure to degradation by various enzymes, or cellular uptake upon contact with surface receptors. Members of the TGFβ family are subject to most of these processes, but they are distinctive in their reliance also on binding proteins as a potent regulatory mechanism for ligand availability and bioactivity. Early in embryogenesis, for example, developmental cues from several of the BMPs and growth/differentiation factors (GDFs) are tightly regulated through interaction with inactivating binding proteins such as chordin, noggin, cerberus, gremlin, and DAN (256, 257).

FS is the longest known and perhaps most extensively studied of these proteins. As noted earlier, FS originally was isolated from ovarian follicular fluid as a protein capable of suppressing FSH secretion from pituitary cells in a manner similar to inhibin (27). Cloning and sequencing (258) showed it to be a monomeric protein unrelated to inhibin, however, and its mode of action became clear with the demonstration of its potent ability to bind activin (13). The high affinity of activin-FS interaction approaches irreversibility due to its slow dissociation rate (43), rendering the bound activin unavailable for binding to its own (activin-RII/RIIB) receptor (259). The bound complex consists of two FS molecules for each activin β/β homodimer (260); the low affinity of inhibin for FS (43) may be due to availability of only a single β subunit in the β/α heterodimer.

The structure of FS is typical of "mosaic" proteins derived from exon shuffling, a mechanism enabling nature to encode a wide range of proteins at considerable savings of genetic material. Following a signal peptide and a 63-residue N-terminal segment, the remainder of the molecule consists of three successive 73-77-residue domains, precisely defined by intron-exon junctions. Clearly related by alignment of their 10-cysteine residues, these distinctive "FS" (FS) domains share about 50% homology in amino acid sequence. FS is variably glycosylated at two N-linked consensus sites within the FS-domain region, but these carbohydrates do not affect activin binding or bioactivity (261, 262).

Two variants of FS are encoded through alternative splicing at the carboxyl-terminus. A 288-residue molecule (FS288) terminates after the third FS domain, whereas a 315-residue form is extended by an extra exon to include a highly acidic 27-residue "tail." Both are capable of binding activin, although according to some reports the affinity of FS288 may be higher than that of FS-315 (261, 263). Secreted FS-315 appears also to be processed by enzymatic cleavage to shorter intermediate forms of 300–303 residues (261, 262). The nature and regulation of this processing has as yet not been defined.

Earlier emphasis on FS as a circulating factor has been largely superseded by evidence for its role as a local cellular regulator. Localization is achieved through potent interaction of FS with cell-surface proteoglycans. This occurs largely through a consensus heparan-sulfate binding site, a cluster of repeated basic (Lys, Arg) residues in a "BBXB" pattern characteristic of similar sites in other proteins, located within the 75–78 sequence in the first of the three FS domains (192, 264). At least one report (265) has shown that, once bound at the cell surface, FS-sequestered activin undergoes accelerated endocytosis and intracellular degradation. Cell surface-bound FS has been postulated to form a barrier or "canopy," limiting approach by exogenous activin of endocrine or even paracrine origin, while allowing access by endogenous, autocrine-derived activin secreted beneath the "canopy." (193, 266).

The FS isoforms differ significantly in their ability to bind to cell surfaces (262, 267). FS288 and the processed form(s) FS-300/303 bind strongly and appear responsible for the local actions of FS, although the actual form(s) of FS that are associated with cell surfaces have yet to be identi-

fied. Full-length FS-315, the predominant circulating form as noted in an earlier section, binds only weakly—possibly because of interference with the heparin-binding site by the acidic C-terminal extension. This accounts, at least in part, for the decreased ability of FS-315 to suppress endogenous activin in pituitary and other cells, as observed initially by Inouye *et al.* (261) and by many others since then. These differences may confer distinct physiological actions based on compartmentalization of FS forms. For example, because FS purified from follicular fluid appears to be predominantly FS-300/303 (261, 262), proteolytic processing of FS315 by individual tissues may be one means by which secreted FS can remain with its tissue source, such as the ovarian follicle, to affect local actions of activin. A corollary to this would suggest that the source of circulating FS, which remains undefined to this day, does not possess this C-terminal proteolytic activity; once the FS protease is identified, analyses of its distribution should help to identify these source(s).

Suppression of pituitary FSH secretion is clearly the "classical" effect of FS and the basis for its most widely used biological assay. Local effects on granulosa cells during follicular development were described in the section "Regulation of Ovarian Follicular Development" and references (17, 191, 268). Although by immunocytochemistry FS is indeed abundant in the pituitary and gonads, it is widely distributed among all tissues in which activin is also present. In fact, the lethal effects found in FS null animals are attributable to skeletal and cutaneous abnormalities that precede any discernible effects on reproductive function (225). FS/activin interactions appear to take place at several steps during the processes of cartilage maturation, endochondral bone formation, and osteoblast formation and bone remodeling (242, 246, 269). A role for FS has been proposed also for such processes as erythroid/myeloid precursor differentiation, pancreatic islet cell function, hepatocellular regeneration, and placental function during parturition (17, 270). Recent evidence (233) suggests an important influence of FS in stimulating renal tubular proliferation during recovery from ischemia and renal failure, counteracting the effects of activin on promoting terminal differentiation. Several of these activities in conjunction with activin action are discussed in more detail in the previous section, "Local Regulation of Activin Action."

FS appears early in *Xenopus* blastula development and has been shown to inhibit the potent mesoderm-inducing effect of activin, a critical step in the induction of neuralization (271). FS is also localized in tissues associated with neural induction (Spemann organizer and notochord), where it may also exert a direct effect on neuralization (272). In higher vertebrates, this may represent a redundant or "backup" system because the FS-null mice lack an obvious neural phenotype (225). It now appears that neuralization may be driven primarily by other TGFβ-related effectors such as BMP-2 and BMP-4 (256, 273), whose regulation by their specific binding proteins represents, as noted already, a system closely replicating the interactions between activin and FS.

The FS-Related Gene Family. FS bears structural as well as certain functional similarities to a number of extracellular matrix proteins involved in cellular regulation and development. The 10-cysteine repeats comprising the majority of the FS molecule were likened initially to the EGF-like sequences found in many proteins, as well as to the Kazal or ovomucoid family of protease inhibitors. With the discovery of similar 10-cysteine sequences in SPARC (osteonectin/BM40), agrin, hevin, and an increasing number of other extracellular matrix proteins, it has become clear that this repeating motif indeed represents a distinct "FS-like module" (274) whose ancestry may date back to splicing of the shorter EGF- and Kazal-type domains. Proteins containing these domains are now often referred to as members of the FS-related protein or FS-related gene family (275, 276). Each FS domain forms an autonomous folding unit, as confirmed by the crystal structure of the single FS domain from SPARC (277), localizing all disulfide linkages exclusively to intradomain cysteines. Aside from the conserved cysteine alignments, the amino acid sequences of the FS domains differ among the various proteins, typically to an extent greater than the 50% identity found among domains within FS itself. Amino acid sequences as well as intercysteine intervals are more highly conserved within some regions than others, features that may be important in maintaining the three-dimensional structure and/or orientation of the domain within the context of the overall molecule.

Despite their outwardly diverse actions, many of these proteins, including FS, share functional properties that are met in differing ways. Almost all are associated with cell surfaces through different mechanisms. Whereas FS recognizes cell-surface heparan sulfate moieties through its consensus sequence in FS domain I, agrin and testican themselves contain glycosaminoglycan side-chains recognizing binding sequences in cell-surface proteins (278, 279). A major form of agrin also contains an N-terminal laminin-binding site that is essential for its association with synaptic basal lamina to induce formation of neuromuscular junctions (280), and the hypothalamic protein X-7365 is anchored directly through a membrane spanning sequence close to the C-terminus (281). Several of these proteins, like FS, have been postulated to exert regulatory effects through the binding of various growth factors or other ligands, either to the FS domains themselves or to other regions of the molecule. Hence, SPARC/BM40/osteonectin has been shown to bind platelet-derived growth factor (PDGF), a cysteine-knot growth factor related to TGFβ, as well as vascular endothelial growth factor and basic fibroblast growth factor, leading to inhibition of the proliferative and angiogenic activities of the respective ligands (282, 283).

Recent structure-activity studies using mutant FS (284), together with an earlier mutagenesis study (261) and analyses of direct binding to activin by synthetic peptides (285), clearly implicate the 63-residue N-terminal segment, pre-

ceding the FS domains themselves, as a critical region for activin binding. Mutation of either of two tryptophan residues, at positions 4 and 36, markedly diminishes activin binding as well as abolishing the biological effects of FS on activin-mediated transcriptional responses and suppression of FSH secretion in cultured pituitary cells (284). On the other hand, mutation of the numerous charged residues in the N-terminal region had little effect. These results suggest that hydrophobic contacts play an important role in FS/activin interaction. Significantly, in the crystal structure of the extracellular region of the activin type II receptor, three hydrophobic residues have been identified as key components of an activin-binding "pocket" (286, 287), implying that FS might neutralize activin by competing with the receptor for hydrophobic sites on the activin molecule.

The N-terminal region, although essential, it is not sufficient for activin binding without participation by one or more of the three FS domains comprising the remainder of the molecule. Assays of a series of domain-deletion mutants indicate that the first and second FS domains, but not the third, are also required for activin binding and FS bioactivity (284). Further studies are required to learn whether determinants in these two domains provide additional direct contacts or augment binding through secondary interactions or conformational influences on the N-terminal domain.

Recently, a closely related homolog of FS, known as FS-related gene product (FLRG; Refs. 275, 288) or FS-related protein (FSRP; 276, 289) has been cloned and localized to a variety of tissues and cell types. It binds activin with an affinity approaching that of FS, and inhibits activin transcriptional activity in reporter assays (276, 288). FLRG/FSRP differs from the extracellular FS-related genes in that its overall gene structure is quite similar to that of FS itself, with a signal peptide, N-terminal domain, two FS domains, and a C-terminal tail domain, all encoded on separate exons. Thus, based on its similar sequence, gene structure, and activin-binding properties, FS and FLRG/FSRP might comprise a separate subfamily of FS-related genes, distinct from the extracellular matrix proteins that possess one or more FS domains but do not appear otherwise to be functionally related to FS. To reduce confusion with other "FS-related" genes, we have referred to FLRG as FSRP in this review.

FSRP differs significantly from FS and the other FS-related genes described above in lacking a consensus heparin-binding sequence or other means for binding to cell surfaces. Accordingly, its ability to suppress FSH secretion in response to endogenously produced activin in cultured pituitary cells is diminished at least 10-fold compared with FS itself (276). This "experiment of nature" emphasizes the importance of the heparin-binding sequence to the ability of FS to neutralize activin of autocrine origin.

Several other intriguing differences between FS and FSRP further suggest that they may not be complete functional homologs. Although FSRP and FS are ubiquitously expressed, their maximum expression takes place in different tissues: Placenta, testis, and cardiovascular tissue are notable for their high content of FSRP, whereas FS is considerably higher in pituitary and ovary. Their intracellular distribution also differs; FSRP, unlike FS, is localized prominently in the nucleus in many cell types, and appears to be secreted only by those cells with the highest levels of FSRP transcription (289). Although a physiological role for FSRP remains to be defined, it may be anticipated that the two molecules mediate distinct biological activities, especially with respect to cellular localization and interaction with exogenous versus endogenous activin. In a broader context, the combination of related but functionally different gene products (FS and FSRP) in short and long forms (e.g., FS288 and FS-315) has interesting parallels with the agrins, in which combinations of variants arising from alternative splicing and expression of distinct gene products (280) results in considerable diversity of localization, tissue distribution and, inevitably, physiological actions.

The close correlation between activin binding, activin transcriptional regulatory activity, and suppression of pituitary FSH secretion among mutant FS (284) continues to support the neutralization of activin as a primary mechanism for the biological actions of FS. However, FS has also been observed to bind other TGFβ-related growth factors, including several of the BMPs and certain members of the GDF family (290–293). Binding affinities for the BMPs are considerably lower than for activin, but local concentrations of FS may be sufficient to enable it to act as a regulator in the numerous systems expressing these factors. Hence, FS has been reported to neutralize BMP-2 and BMP-4 action in embryogenesis and may influence cartilage formation, maturation, and bone development through interactions with BMP-7 and others (290, 294), including the newly described BMP-11 that binds to FS but not noggin or chordin (291). A recent report (188) shows that BMP-6 and -7 and, potentially, their interactions with FS or FSRP, may even be components in the control of pituitary FSH secretion as discussed in the section "Regulation of Activin Activity."

Certain interactions may not result in neutralization of the ligand, perhaps because of the involvement of different contact sites than those found between activin and FS. Tripartite complexes involving FS, ligand, and receptor have been proposed, for example, that may indirectly modulate or even enhance BMP or GDF activity (295, 296). Finally, returning to the pituitary as a prototype system, FS may modulate FSHβ transcription, FSH heterodimer secretion, and regulation of GnRH receptor levels through different mechanisms, especially should its actions prove to involve other ligands besides activin, or even some as-yet-undefined FS "receptor." Nevertheless, it remains clear that together, FS and FSRP act locally or (in the case of FSRP) even intracellularly to regulate the activity of activin, and perhaps other TGFβ superfamily members. Using a combination of genetic, biochemical, and molecular strategies, both the mechanism(s) and physiology of these actions should become better defined over the next 5 years, perhaps resulting

in delineation of novel therapeutic applications of FS, FSRP, or their antagonists.

Intracellular Modulators of Activin Action

At the time of the last contribution to this series, the skeleton of activin's signal transduction machinery had been identified, including membrane receptors, second messengers, and transcriptional activators. Since that time, a great deal has been learned about this system, uncovering a complex web of modulators, inhibitors, co-activators, and interacting signaling systems, the physiological significance of which with respect to activin actions remains to be demonstrated. In addition, there is a great deal of promiscuity between TGFβ superfamily members, further complicating the task of unraveling the molecular basis for the signal transduction of activin. On the other hand, this sharing of molecular components between ligands means that to some degree, pathways identified for one ligand are often relevant and even functional for others. This is especially true for activin and TGFβ, where common signaling components and modulatory proteins make it essential to examine both systems. Fortunately, this active area has spawned numerous reviews, which emphasize both intricate pathways as well as common themes (297–300). We will therefore attempt to summarize recent discoveries relevant to understanding activin's signal transduction pathways, paying particular attention to proteins that can modulate this signal, or to intersections with other signaling pathways that might be important for understanding activin's actions.

Basics of Activin Signaling. Activin signal transduction is mediated by a heterotetrameric receptor complex consisting of two ligand-binding type II receptors and two signal transducing type I receptors (297). Upon ligand binding, the type II receptor phosphorylates the type I receptor on multiple serine and threonine residues within a unique GS-rich motif located just N-terminal to the kinase domain. Two type II receptors have been identified that bind activin with high affinity and specificity, known as ActRII and ActRIIB. Similarly, two type I receptors appear to interact with the activated type II receptors to transduce activin signals, including ActRI (ALK2) and ActRIB (ALK4), although it seems that ActRIB is the primary activin effector (301, 302).

It is now evident that different ligands in this family may signal through the same receptors, creating a level of redundancy that obscures specificity. For example, both BMP-2 and -7 may signal via ActRII or ActRIIB in combination with BMPRI or BMPRIB whereas Nodal shares with activin both Type II receptors as well as ALK4 (256). In these cases, specificity may be provided by coreceptors and other accessory proteins, but the nature of these molecules and their function in TGFβ family signaling are only now being uncovered (see below). Furthermore, it is still not clear whether specific receptor isoform combinations mediate distinct activin responses *in vivo*.

To transmit the signal intracellularly, type I receptors activate a member of the SMAD family, which then translocates to the nucleus. Based on structural and functional characteristics, the SMAD family is divided into three distinct classes: receptor-regulated SMADs (R-SMADs), common-mediator SMADs (Co-SMADs), and inhibitory SMADs (I-SMADs). R-SMADs include SMAD2 and 3, which are specifically recognized by activin and TGFβ type I receptors, and SMAD1, 5, and 8, which are used by BMP receptors (256, 303). The R-SMADs contain two conserved domains that form globular structures separated by a linker region (304, 305). The N-terminal MH1 domain exhibits DNA-binding activity, whereas the C-terminal MH2 mediates protein-protein interaction, facilitates translocation into the nucleus, and regulates transcription. Phosphorylation of the C-terminal SSxS sequence by activated type I receptors appears to release these two domains from an autoinhibitory configuration, leading to R-SMAD activation (298). Once activated, R-SMADs associate with one of two known co-SMADs, including SMAD4 and SMAD4b/SMAD10, with the complex then translocating to the nucleus (306–308). The Co-SMADs have an MH1–linker–MH2 domain structure similar to the R-SMADs but lack the SSxS sequence and are therefore not directly phosphorylated by type I receptors. It has been suggested that they are required for the formation of functional transcriptional complexes rather than for mediating nuclear translocation itself (309). I-SMADs are distinct from the other two groups in that they have a conserved MH2 domain but diverse N-terminal domains and no SSxS motif. Thus, they are capable of forming stable associations with the type I receptors but cannot be activated, thereby preventing activation of the R-SMADs. There are two known vertebrate I-SMADs, including SMAD6, which represses the BMP pathway and SMAD7, which inhibits signaling in the activin/TGFβ pathway (see below).

Once in the nucleus, both R-SMAD and Co-SMAD may participate in DNA binding and recruitment of transcriptional cofactors. DNA binding is mediated by a highly conserved β-hairpin structure that protrudes from the surface of the MH1 domain and binds in the major groove (304). α-helix 2, which is located n-terminal to the β-hairpin element, may also participate in DNA binding, perhaps conferring differential specificity among R-SMADs (310). Interestingly, the most common splice form of SMAD2 lacks DNA-binding activity because of an insert located next to the β-hairpin. SMAD binding elements, which contain the consensus motif GTCT (or AGAC; Refs. 304, 311), are often present in promoters of TGFβ, activin or BMP target genes (reviewed in Ref. 312), but their presence is not obligatory (313). BMP-activated SMADs have also been shown to bind GC-rich motifs in the promoter of BMPs responsive genes (314, 315). Nonetheless, the short recognition sequence of SMAD binding elements and their low affinity for direct SMAD binding suggest that cooperation with other transcription factors may be required for activation of specific target genes (304).

Of these transcriptional co-activators, one of the best-characterized examples is forkhead activin signal transducer (FAST-1, also known as FoxH1), a winged helix forkhead transcription factor, that regulates activin-dependent induction of the *Mix.2* gene in Xenopus and *goosecoid* in mouse (316, 317). FAST-1 binds constitutively to specific activin response elements in target genes but cannot activate transcription by itself. Stimulation by activin or by closely related ligands in the activin/TGFβ subgroup results in the formation of complexes that contain FAST-1, SMAD2, and SMAD4. SMAD2 associates directly with FAST-1, whereas SMAD4 contributes additional DNA binding specificity and transcriptional activation functions to the complex (318). SMAD3 can replace SMAD2 in transcriptional complexes at the *Mix.2* activin response element (319) but oppose it in the *goosecoid* gene (313). Differential roles of SMAD2 and SMAD3 were also observed in human keratinocytes, where TGFβ stimulates SMAD2 and SMAD3 to comparable extents while activin predominantly activates SMAD3 (320). Moreover, recent studies in fibroblasts derived from mouse embryos deficient in SMAD2 or SMAD3 have identified SMAD2 and SMAD3 specific functions as well as R-SMAD-independent responses to TGFβ (321).

Additional DNA binding partners of SMADs have been reported to regulate transcription of activin/TGFβ target genes. For example, SMAD2 and/or SMAD3 can associate with c-Jun/c-Fos, ATF2, TFE3, PEBP2/CBF, and with the vitamin D receptor (reviewed in Refs. 299, 312, 322). In many situations, these partners are also activated by other signaling pathways, providing a mechanism for integrating signals originating from a variety of sources to regulate cellular response (see below). Moreover, once recruited to specific elements, both SMAD2/3 and SMAD4 can activate transcription by recruiting additional co-activators that have histone acetyl transferase activity such as CBP/p300 (323–326). Alternatively, they can recruit corepressors such as TGIF or Ski family members, which in turn bind histone deacetylases (327–329). Thus, SMADs can positively or negatively regulate transcription of specific genes in response to activin/TGFβ signaling.

Modulation of Activin Signaling by Accessory-Receptors. Conversion of the activin-signaling pathway to an inhibin mediated activin-suppression pathway by the recently identified inhibin coreceptors (see above) represents one example of activin signal modulation by accessory receptors. Another candidate accessory receptor is endoglin (CD105), which is a homodimeric transmembrane glycoprotein expressed at high levels on endothelial cells. Endoglin is similar in sequence and structure to betaglycan, especially in the cytoplasmic and transmembrane regions. When overexpressed, endoglin can interact with a variety of TGFβ family ligands, includingTGFβ1 and 3, activin-A, BMP-7, and BMP-2, but only when they are complexed to their respective Type II receptors (330). Endoglin has also been shown to antagonize TGFβ1 in multiple *in vitro* bioassays (331–334). Moreover, accumulating genetic data suggests a connection between endoglin and the type I receptor ALK1 because mutations in both genes lead to similar defects in vascular formation (reviewed in Ref. 335). The physiological ligand and the type II receptor partners of ALK1 are still controversial, but *in vitro* studies as well as in situ hybridization studies have suggested ALK1 may bind TGFβ as well as activin (336–338).

Another membrane-associated group of proteins that could modulate activin signaling is known as the EGF-CFC family, which contain two conserved domains: a variant of the EGF domain, and a unique cysteine-rich domain called CFC (Cripto, FRL-1, and Cryptic; reviewed in Ref. 339). Two members of this family have so far been identified in mammals, including Cripto and Cryptic. Genetic studies in zebra fish and mice have revealed that Cripto is actually a coreceptor that converts the activin signaling system to a nodal signaling pathway (339). This is accomplished through an interaction between Cripto and the activin type I receptor ALK4 via its conserved CFC domain. This interaction is necessary for Nodal/ActRII/ALK4 complex formation and for SMAD2 activation (340). Interestingly, Cripto-null mice demonstrate most but not all the defects observed in Nodal-disrupted mice, suggesting some Cripto-independent Nodal activity (341). This may be mediated by nodal-forming heterodimers with BMPs (similar to inhibin/activin), thereby having antagonistic effects at the level of the dimeric ligand production and/or receptor binding (340). Cripto also has been implicated in carcinogenesis, but the exact mechanism of its action in this situation is not clear (341). Moreover, the role of EGF-CFC in activin signaling has not yet been explored.

BMP and activin membrane-bound inhibitor (BAMBI) is a transmembrane protein that is related to TGFβ-family type I receptors but lacks an intracellular kinase domain (342). BAMBI can block both activin and BMP signaling by forming stable associations with all type I receptors except ALK2, thereby preventing their activation. Although the biological activity of BAMBI *in vivo* remains to be determined, it is co-expressed with the ventralizing morphogen BMP-4 during Xenopus embryogenesis and requires BMP signaling for its expression (342). In addition, expression of the human BAMBI ortholog gene (*NMA*) was identified as a downregulated gene in metastatic melanoma cell lines (343). These preliminary observations suggest that BAMBI will have important regulatory functions in both the activin and BMP signal transduction pathways and that loss of function leads to pathophysiological outcomes.

Intracellular Modulators of Activin Signaling. Considering the critical role SMADs play in transmitting the intracellular signal of TGFβ family ligands, it is not surprising that cells regulate the availability of SMAD at multiple levels. For example, SMAD2 access to the type I receptor is modulated by a protein named SMAD anchor for receptor activation (SARA; Ref. 344), which binds unphosphorylated SMAD2, thereby masking its nuclear localization signal and maintaining a cytoplasmic distribution.

Upon receptor activation, SARA interacts with the type I receptor to facilitate SMAD2 phosphorylation and activation (344). As a result, SMAD2 dissociates from SARA and complexes with SMAD4, after which the complex translocates to the nucleus. In addition to regulating the activation of SMAD2, SARA can also regulate its subcellular localization. SARA contains a conserved zinc finger-like structure known as FYVE domain, which allows SARA to associate with the endosomal membrane. Thus, this FYVE domain is required for proper localization of SARA/SMAD2 complexes in punctuate regions that also contain the receptors (344) because mutations in this domain inhibit SMAD2-dependent transcriptional responses. Although SARA can also bind SMAD3, the significance of this interaction for TGFβ signaling has been questioned (345). It also appears that FYVE domain-containing proteins are involved in endocytosis and vesicular trafficking (346), raising the possibility that activated receptor complexes formed at cell surface may have to internalized in order to contact R-SMADs, a process that is regulated by SARA and other FYVE motif proteins. Indeed, another FYVE domain protein, HGS, which is involved in membrane trafficking and vesicle fusion, also binds SMAD2/3 and cooperates with SARA to stimulate activin receptor-mediated signaling (347). Disruption of the HGS gene in mice is embryonic lethal, and isolated mutant cells show compromised activin and TGFβ transcription responses. These preliminary results suggest that SARA and other FYVE domain proteins are critical intracellular regulators whose biological role in activin signaling remains to be determined.

Another suggested mechanism for regulating SMAD distribution and access to receptors is the microtubule network (MT). Both SMAD2/3 and SMAD4 interact with the MT in a ligand-independent manner and are released upon receptor activation (348). Drugs that destabilize MT also disrupt its association with SMADs, causing an increase in ligand-induced SMAD2 phosphorylation and transcriptional response. Thus, MTs may serve as a cytoplasmic sequestering network for regulating SMAD activity.

Ubiquitination of R-SMADs represents another mechanism for modulating both intensity and duration of the activin-mediated signaling response. Activated SMAD2 that accumulates in the nucleus is specifically targeted by the E2 enzymes UbcH5b/c for proteolytic degradation by the ubiquitin/proteasome pathway (349). It is still unclear whether other SMADs are also degraded in the nucleus by this pathway. Interestingly, a distinct ubiquitination process has been shown to regulate the basal concentration of BMP-responsive R-SMADs. This process is mediated by the specific E3 ligase Smurf-1, which preferentially recruits cytoplasmic SMAD1 and 5 to the ubiquitin conjugation machinery (350). A similar degradation mechanism for SMAD2/3 has yet to be identified.

As we have alluded to above, I-SMADs play a major role in regulating receptor mediated R-SMAD activity. SMAD7 inhibits primarily the activin and TGFβ-signaling pathways whereas SMAD6 inhibits BMP signaling (reviewed in Refs. 256, 297, 303). I-SMADs can be found in both the cytosol and nucleus, and their expression increases rapidly after induction by TGFβ family ligands. For example, activin-A has been shown to rapidly and transiently increase SMAD7 mRNA levels in rat anterior pituitary gonadotroph and corticotrope cell lines, and ectopic expression of SMAD7 blocked reporter transcription in these cells (186).

I-SMADs exert their antagonistic effects using a variety of mechanisms. When ectopically expressed both SMAD6 and SMAD7 can bind directly to type I receptors, thereby blocking their interaction with R-SMADs (256). However, it is not clear whether I-SMADs also function in this way at physiological expression levels. For example, when expressed at low levels, SMAD6 inhibits BMP signaling by competing with SMAD4 for binding to activated SMAD1 rather then by blocking receptor-mediated phosphorylation of SMAD1 (351). I-SMADs may also act in the nucleus at the transcription level. Accordingly, both SMAD6 and 7 have been shown to block SMAD1-induced transcriptional activity by recruiting histone deacetylases in a ligand-dependent manner (352). Moreover, SMAD6 has also been shown to complex with the corepressor Hoxc-8 to promote direct binding of SMAD6 to DNA through its MH1 domain (353).

Additional mechanisms for I-SMAD antagonism of activin signaling may involve increasing receptor turnover rate. SMAD7 has been shown to recruit Smurf1 and Smurf2 E3 ubiquitin ligases, thereby targeting the TGFβ type I receptor for degradation by the proteosomal and lysosomal pathways (354, 355). Interestingly, at low expression levels, SMAD7 has been reported to inhibit TGFβ-induced reporter transcriptional activity but had little effect on TGFβ-induced growth inhibition (356). Similarly, SMAD7 has been shown to antagonize activin-induced erythroid leukemia cell differentiation and reporter transcriptional activation when both were mediated by ActRI but not by ActRIB (357). These observations suggest that TGFβ and activin can signal via more then one pathway and that SMAD7 may interfere with these pathways via different mechanisms depending upon its expression level. Clearly, the SMAD second messenger system can be modulated at a variety of levels, the sum of which appear to modulate the magnitude and/or duration of activin signaling.

Modulation of Activin/TGFβ Signaling by Other Pathways. Recent reports have now identified intersections between the SMAD pathway and signaling cascades for other growth and differentiation factors (reviewed in Refs. 256, 298, 358). Although these studies are still in their infancy, it is now clear that this will be an emerging theme for the next few years and should be included among the activin modulation mechanisms. It is also important to realize that for the most part, these pathway intersections had been demonstrated primarily for the TGFβ signaling

system but are undoubtedly applicable to activin and BMP signaling as well.

The INF-γ/STAT pathway has been demonstrated to modulate the TGFβ/SMAD pathway by rapid induction of SMAD7 synthesis. Increasing levels of SMAD7 in turn suppress TGFβ-induced SMAD3 phosphorylation and nuclear accumulation (359). Similar mechanisms have been shown to mediate the antagonist effect between NF-kappaB pathway and TGFβ/activin/SMAD signaling (360, 361).

The MAP kinase pathway may modify SMAD activity negatively or positively depending on physiological context. Ras-activated ERK protein kinases have been shown to directly interfere with SMAD-mediated responses by phosphorylation of R-SMADs in the linker region, preventing their nuclear accumulation (362, 363). Furthermore, protein kinase C, acting downstream of tyrosine kinase receptors, can directly phosphorylate SMAD2 and 3 in their MH1 domain, abrogating DNA binding and leading to downregulation of the growth inhibitory and apoptotic actions of TGFβ (364). In contrast, other studies have shown that receptor tyrosine kinase-mediated signals induce rapid phosphorylation and nuclear translocation of SMAD proteins by kinase(s) downstream of MEK1 (65,366).

Calmodulin, the primary mediator of calcium signaling, has been demonstrated to interact with R-SMADs and block activin induced transcription in a calcium-dependent manner (367), as well as to decrease SMAD2-dependent effects while increasing SMAD1 actions in Xenopus embryos and explants (368). In addition, an interaction between TGFβ and estrogen receptor signaling systems mediated via SMAD3 was recently described (369). Taken together, these early observations suggest that the cellular integration of multiple signaling pathways will ultimately dictate the type, duration, and magnitude of a particular biological response. It will thus be critical to investigate the mechanism(s) underling pathway integration as this information may have significance for designing pharmacological agents to manipulate these pathways.

Modulators of Receptor Function. WD proteins contain highly conserved repeating domains of approximately 40 amino acids that typically end in tryptophan-aspartate (WD), which are thought to mediate protein-protein interactions . They regulate multiple cellular functions, including cell division, cell-fate determination, gene transcription, transmembrane signaling, mRNA modification, and vesicle fusion (370). Three WD proteins, including TGFβ-receptor interacting protein-1 (TRIP-1), serine-threonine kinase receptor-associated protein (STRAP), and the B-alpha regulatory subunit of protein phosphatase 2A (B-α PP2A), have been identified so far as TGFβ receptor-interacting proteins. Little is known about their role in TGFβ signal transduction, but they may be homologous in function to SH2 domain-containing proteins in the tyrosine kinase receptor pathway (371).

TRIP-1 interacts with and is phosphorylated by TGFβ type II receptor in a ligand independent manner. It can also be coprecipitated, both *in vivo* and *in vitro*, with ligand-bound complex of type I and II receptors but does not interact directly with the type I receptors (372). Interestingly TRIP-1 overexpression can selectively represses TGFβ-induced transcription from a reporter gene but failed to antagonize its growth inhibition activity (373). STRAP on the other hand, associates with both types of TGFβ receptors in a ligand-independent manner. When overexpressed, it can modulate TGFβ signaling, perhaps by recruiting SMAD7 (374). B-α PP2A, associates with, and is phosphorylated by activated TGFβ type I receptors (375). Like TRIP-1, B-α exhibits selectivity with respect to its effects on signaling, but with the opposite specificity. It regulates the antimitogenic signaling pathway of TGFβ but does not have an effect on reporter gene transcription. These specific effects may be manifested via the phosphatase 2A activity (375). Although TRIP-1, B-α PP2A, and STRAP have been shown to function in TGFβ signaling, it is reasonable to assume that these or related WD proteins might have similar role in activin signaling.

Another group of proteins that may modulate the activity of activin and TGFβ receptors are known as PDZ domain proteins. The PDZ domain is part of a molecular scaffold that holds multiprotein signaling complexes together. It is generally believed that the role of PDZ domains is to position target ion channels, receptors, or other signaling molecules in correct spatial arrangement in relation to each other and to specialized regions of the cell (reviewed in Refs. 376, 377). Activin receptor interacting protein 1 (ARIP-1) is a PDZ protein that specifically recognizes activin type IIA receptors and is expressed mainly in the brain. Interestingly, ARIP1 also interacted with SMAD3, and when ectopically expressed inhibits activin-induced and SMAD3-induced transcription in activin-responsive cell lines (378).

A second PDZ domain-containing protein that interacts with TGFβ family receptors is GAIP-interacting protein, C terminus (GIPC). GIPC binds a Class I PDZ binding motif in the cytoplasmic domain of betaglycan (type III TGFβ receptor) and facilitates its expression at the cell surface. The increased expression of betaglycan mediated by GIPC enhances cellular responsiveness to TGFβ both in terms of inhibition of cell proliferation and in SMAD-mediated gene transcription (379).

TβRI-associated protein-1 (TRAP-1) was first identified based on its ability to specifically interact with the activated form of TGFβ type I receptor (380). More recently, TRAP-1 has been shown to prefer inactive heteromeric TGFβ and activin receptor complexes (381). Upon ligand activation, TRAP-1 is released from the receptor complex and interacts with SMAD4. Mutated constructs of TRAP-1 inhibit TGFβ signaling and diminish the interaction of SMAD4 with SMAD2. Thus, TRAP-1 may act as a chaperone for SMAD4, bringing it into the vicinity of the receptor complex, thereby facilitating formation of activated SMAD4/R-SMAD complexes (381).

Alternative Signaling Pathways for Activin/TGFβ. Although SMADs are the primary second messengers of the TGFβ superfamily, there is accumulating evidence for a role of other signaling pathways in mediating at least some of the diverse biological responses. Many studies have implicated TGFβ superfamily members in activation of MAP kinase pathways (298,382). Although the initial steps coupling the receptor serine kinase to the mitogen-activated protein kinase (MAPK) system are still not clear, an impotent role has been proposed for the MAPKKK family member TAK1 (TGFβ-activated kinase) as an upstream mediator of the signal cascade (383–386). Signaling via TAK1 usually results in activation of AP-1 or CRE-regulatory transcription complexes, which in turn can cooperate with activated SMADs to mediate transcription at defined promoters as well as cell proliferation (387). Moreover, both TGFβ and activin have been shown to induce TAK1, leading to activation of the stress-activated kinase p38 and the transcription factor ATF2 (388, 389).

Conclusions and Future Directions

It seems clear from this review that our understanding of the physiology of inhibin, activin, and FS is further developed than the determination of molecular pathways by which these actions are mediated. Moreover, it is also becoming clear that the original model of TGFβ superfamily signaling being mediated solely by the SMAD second messenger pathway is somewhat naive. Thus, it is likely that the most significant advances in the near future will come from a greater and more sophisticated understanding of signaling pathways and components, how they interact, and which modulators have relevance for normal physiological processes, as well as in disease states.

Another theme that emerges from this review is that it might be appropriate to rearrange the regulatory triangle represented by inhibin, activin, and FS. From a reproductive perspective, inhibin was the proband of this whole group of nonsteroidal regulators, stimulating more than half a century of research that ultimately lead to the discovery of activin and FS. Similarly, in the physiological arena, inhibin seems to be primarily an endocrine hormone with at least one clearly defined endocrinological action that has been verified using a host of classic ablation-replacement strategies. On the other hand, activin and FS appear to be more local in action, perhaps with paracrine or even autocrine actions as their primary biological function(s). These types of actions are harder to prove, so at present, we still don't really know the biological role(s) of these two proteins, although they are clearly critical for both development and reproduction. Nevertheless, the discovery of inhibin co-receptors that can divert the activin signaling pathway to promote their activin-antagonist effects, along with our new understanding of how effectively and completely FS can inhibit activin signaling, lead us to a new paradigm that might be useful for designing and testing new models in the next few years. We suggest that among these three molecules, activin is the main driving force, produced either constitutively or with some minor regulation, and acting in a largely autocrine or paracrine mode. This activin signaling is modulated by a variety of factors acting at different steps. Prime among these factors are the extracellular modulators, inhibin and FS, which together influence how much activin can contact its receptor, and/or how effectively the ligand-receptor complex can initiate its signaling cascade. Once an activin signal is initiated, however, regulation becomes intracellular, involving interaction with modulatory proteins, other signaling pathways, and transcriptional enhancers and repressors, the result of which is an exquisitely maintained and constantly appropriate level of activin signaling.

What happens when this precise regulation of activin action breaks down? We now have numerous examples, but it is here that the genetic models provide the best information, if not definitive answers. For example, elimination of inhibin biosynthesis and consequent elevation of activin production and signaling can lead to very aggressive and lethal gonadal and adrenal tumors, while loss of activin or FS is perinatally lethal. It is therefore no wonder that nature has evolved this multilayered control system to insure that in most circumstances, cells get only the activin stimulation they need. Moreover, this concept is probably true for most other TGFβ superfamily members, suggesting that by integrating and generalizing discoveries from one subfamily to the others, we can expect even more expeditious unraveling of this entire system. Thus, the paradigm for the new millennium may provide a framework whereby interactions between and among the entire TGFβ superfamily might be integrated to provide a more accurate definition of the biological actions of each member.

However, to get there, we first need to identify and understand how the components of this system fit together. For these studies, models such as those developed to study TGFβ might be extremely useful. Fibroblasts derived from mouse embryos deficient in either SMAD2 or SMAD3 were isolated and cultured as cell lines (321). This system then allowed the dissection of which responses to TGFβ (and by extension, activin or nodal, etc) are mediated by which second messenger. Extending this to other parts of the system which appear to be shared by more than one TGFβ superfamily member, or perhaps by isolating cell lines from embryos with multiple genes knocked out, it should be possible to determine the specific function of each element in the signaling cascade at the cellular level. This may help answer the very perplexing conundrum of how hormone-specific responses are mediated in cells when components of signaling systems are shared between two or more members.

The next 5 years look both promising and challenging for deciphering the physiologic and pathophysiologic actions of activin, inhibin, and FS, as well as the whole TGFβ superfamily. Recent technological innovations, including more sophisticated genetic approaches, continuously improved gene and protein chip technology, RNA interference

methods, and other yet to be described methodological discoveries suggest that the best is yet to come.

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