The Local Control of the Pituitary by Activin Signaling and Modulation

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Abstract: The pituitary gland plays a prominent role in the control of many physiological processes. This control is achieved through the actions and interactions of hormones and growth factors that are produced and secreted by the endocrine cell types and the non-endocrine constituents that collectively and functionally define this complex organ. The five endocrine cell types of the anterior lobe of the pituitary, somatotropes, lactotropes, corticotropes, thyrotropes and gonadotropes, are defined by their primary product, growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH) and follicle stimulating hormone (FSH)/luteinizing hormone (LH). They are further distinguishable by the presence of cell surface receptors that display high affinity and selectivity for specific hypothalamic hormones and couple to appropriate downstream signaling pathways involved in the control of cell type specific responses, including the release and/or synthesis of pituitary hormones. Central control of the pituitary via the hypothalamus is further fine-tuned by the positive or negative actions of peripheral feedback signals and of a variety of factors that originate from sources within the pituitary. The focus of this review is the latter category of intrinsic factors that exert local control. Special emphasis is given to the TGF-β family of growth factors, in particular activin effects on the gonadotrope population, because a considerable body of evidence supports their contribution to the local modulation of the embryonic and postnatal pituitary as well as pituitary pathogenesis. A number of other substances, including members of the cytokine and FGF families, VEGF, IGF1, PACAP, Ghrelin, adenosine and nitric oxide have also been shown or implicated to function as autocrine/paracrine factors, though, definitive proof remains lacking in some cases. The ever-growing list of putative autocrine/paracrine factors of the pituitary nevertheless has highlighted the complexity of the local network and its impact on pituitary functions.

Keywords: Pituitary, gonadotrope, FSHbeta, LHbeta, gonadotropin, activin, inhibin, follistatin, bone morphogenetic proteins, FOXL2, BPES, Smad.

A BRIEF PERSPECTIVE OF THE PITUITARY AND TGF-β LIGANDS

The historical view of the pituitary as the 'master' endocrine gland derives from the broad spectrum of physiological and homeostatic processes that are controlled and/or influenced by the actions of hormones produced by this organ. Some of the prominent actions of anterior pituitary hormones include the anabolic and growth-promoting actions of growth hormone (GH), the control of sexual maturation and fertility by the gonadotropins (LH and FSH), adaptation and responses to stress and immune activation via ACTH, the effects of PRL on lactation and the critical role of TSH on thyroidogenesis and thyroid function [1]. Appropriate control of the endocrine pituitary cell types that specialize in producing these hormones is therefore critical for survival and general health. This control is achieved by the coordinated actions of central inputs, via the actions of hypothalamic peptide hormones including GHRH, somatostatin, GnRH, CRF, TRH as well as non-peptide inputs such as dopamine, and a variety of peripheral feedback signals [2]. The actions of these extrinsic control signals are further influenced by the immune system through the direct or indirect actions of cytokines, and the variety of autocrine and/or paracrine factors that are produced locally by both the endocrine and non-endocrine cells of the pituitary [3]. Appropriate pituitary function might also be achieved through processes designed to replenish, expand or maintain population sizes [4-6]. A genetic lineage tracing strategy has identified a population of nestin-positive stem-like cells of the adult mouse pituitary capable of differentiating into all pituitary cell lineages [7]. Stem-like and sphere-forming cells of mouse and human pituitaries have been enriched and shown to express embryonic pituitary cell markers and retain proliferative potential [8-11]. The human pituitary has been shown to contain populations of cells that display stem/progenitor characteristics, retain neoplastic potential and express markers of self-renewal [12]. The extent to which these cells contribute to the maintenance of pituitary integrity and functionality *in vivo* has yet to be validated. More than likely, the regulation of the pituitary cell populations is achieved by the combinatorial information supplied by all of these mechanisms, context-dependent alterations in each population as well as a variety of genetic and epigenetic factors that dictate cellular behaviors.

This review highlights key features of the autocrine/paracrine control of the differentiated pituitary, with a primary focus on the actions of activin and its antagonists on gonadotropes. The function of these cells is critical for normal reproductive function as they are the

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source of LH and FSH, which are heterodimers of the unique LHβ and FSHβ, respectively, and the αGSU subunit shared by both. Members of the TGF-β family play prominent and diverse roles in the control of many processes including cell growth, differentiation and apoptosis, proliferation and differentiation of embryonic stem cells, maintenance of homeostatic mechanisms, immune responses as well as tissue remodeling and repair [13-15]. Further validation for the importance of this family comes from observations showing associations between deregulated TGF-β ligand signaling and diseases, cancers and cancer metastasis [16]. Research since the initial characterization of inhibin and activin as FSH-inhibiting and -releasing factors, respectively, has led to insight into their significance and identified the contribution of several other members of the TGF-β family of ligands in the pituitary [17]. The TGF-β ligands have important roles in the postnatal as well as the embryonic pituitary. Several members of this family exert cell type specific effects and are implicated as components of the autocrine/paracrine network of the adult anterior pituitary [17]. During pituitary organogenesis, opposing BMP-4 and BMP-2 gradients are critical for survival and proliferation of the pituitary progenitors and for dorsal-ventral patterning of the mouse pituitary pouch [18, 19]. Such signals might also be critical for the maintenance of the progenitor/stem cell niche of the postnatal pituitary, as they are in other tissues [14]. Growing evidence further implicates the intra-pituitary activin/follistatin network as a critical checkpoint for pathogenic mechanisms that lead to pituitary cell proliferation and tumor formation [20-22].

Inhibins were first characterized as gonadal feedback modulators with inhibitory effects on FSH secretion from preparations of rodent pituitary cells [23]. The analysis of side-fractions of the purified inhibin preparations subsequently led to the identification of activins and the realization that activins and inhibins are members of the TGF-β family and function antagonistically to stimulate and inhibit FSH secretion, respectively [23]. The existence of a non-steroidal feedback signal originating from the gonads had been anticipated from multiple *in vivo* and *in vitro* studies and the demonstration that charcoal-treated follicular fluid suppressed the secondary FSH rise [23]. Similarly, the concept of the existence of an FSH-releasing factor, other than hypothalamic GnRH, was developed based upon many observations including the ovariectomy-induced rapid initial rise in circulating FSH without an accompanied change in LH [23]. Inhibin and activin fulfilled the criteria as FSH-inhibiting and -stimulating factors, respectively [23].

Numerous studies utilizing genetically modified rodent models and also guided by phenotypic observations of pituitary anomalies in human patients have since validated these initial observations of activin and inhibin action and expanded the scope of their targets and actions [24-27]. These studies have also led to the realization that additional members of the TGF-β family might also have cell type specific roles in the pituitary. Variable effects of BMPs on FSH secretion and FSHβ expression have been reported [28-30]. Inhibitory effects of BMP-2/4 on the transcription of proopiomelanocortin (POMC) in corticotropic AtT20 cells seems to be mediated through interference with Pitx and/or Tpit and involve ALK3/6 type I receptors [31]. The extent to which member of the bone morphogenetic protein (BMP) family play a significant role in the postnatal pituitary still remains an open question but they are implicated in pituitary tumorigenesis [32]. The presence of transcripts for BMP receptors and several BMP forms, including BMP-6, -7 and -15 in the mouse [33] and GDF9 in the human pituitary [34], is consistent with the possibility that they act locally. TGF-β1 and -β3 are expressed in the pituitary and act through type II and type I TGF-β receptors expressed on lactotropes to regulate PRL expression and the growth of this population [35]. Studies on rat anterior pituitary cells have shown that whereas TGF-β1 from lactotropes works in an autocrine manner to suppress PRL expression, TGF-β3 mediates the mitogenic actions of estrogen on lactotropes through a paracrine action mediated by basic fibroblast growth factor released from folliculostellate cells [36]. Recent data suggest that lactotropes are also targets of BMP-4 and activin [37]. Studies have further suggested that the Smad-menin association downstream of activin signaling participates in the control of normal cell growth and prevention of tumor cell growth [38].

SIGNALING MECHANISMS USED BY THE TGF-β FAMILY OF LIGANDS

Ligands of the TGF-β family signal through two types of transmembrane receptor serine/threonine kinases (RSK), type I (collectively referred to as ALKs, activin receptor-like kinases) and type II receptors [13]. Downstream signaling is initiated upon ligand binding to both receptors and the formation of an active heteromeric complex, several of which have been validated by recent crystallographic studies [39-41]. An added complexity is generated by the ability of each ligand to interact with more than one receptor and, conversely, of each receptor type to display sufficiently high affinity for more than one ligand, at least under appropriate circumstances [13]. Ligand-receptor choices are ultimately dictated the availability of a particular receptor type at a given site in addition to the structural features that permit the formation of high affinity ligand-receptor complexes. In the case of TGF-β isoforms or activins, high-affinity ligand binding to the respective type II receptor is a critical event that establishes the interface for type I receptor binding, explaining the low or negligible affinity of either ligand for type I receptors [13]. By contrast, BMPs seem to interact with their respective type I and type II receptors with comparable affinity and, for this ligand subfamily, the type I receptor seems to make the major contribution for high affinity binding [13, 42].

Activins transmit their signals through two of the five mammalian type II receptors known to exist, ActRII and ActRIIB, and one of seven type I receptors, ActRIB/ALK4 [13]. ALK4 is the primary type I receptor used by dimeric activins but recent evidence suggests that activin B also signals through ALK7 [43]. The TβRII and TβRI/ALK5 combination is the primary pair used by TGF-β isoforms although recent evidence suggests that TGF-β engages and signals through other type I receptors under certain circumstances [44, 45]. The BMP and GDF subset of the TGF-β family, on the other hand, can signal through ActRIIA, ActRIIB or BMPRII in combination with BMPRIA/ALK3 or BMPRIB/ALK6 but also through additional type I receptors [42, 46].

The cytoplasmic domains of both type I and type II receptors possess serine/threonine kinase activity and also serve as docking stations [46]. According to the currently accepted model for receptor activation, the formation of the heteromeric ligand-receptor complex brings the cytoplasmic domains of the two receptor types into close proximity to each other, thereby allowing the constitutively active type II receptor to phosphorylate key residues within a glycine- and serine-rich (GS) domain of the type I receptors [46, 47]. The phosphorylated type I receptors are then able to transiently recruit and phosphorylate a class of signaling proteins known as Smads [46]. The Smad pathway is considered to be the major but not the only mode of signaling by the TGF-β family of ligands [48]. Moreover, there is considerable crosstalk between Smad and non-Smad pathways [48]. The Smad family can be generally grouped into subcategories based upon whether they are receptor-activated (pathway-restricted, R-Smads) or not (common mediator, co-Smad), whether they serve a modulatory role (inhibitory, I-Smads) and by the identity of the ligands that induce their phosphorylation [46]. Activins and TGF-β isoforms signal primarily via Smad2 and/or Smad3 [48]. BMP signaling, on the other hand, occurs through activation of Smad1, 5 and/or 8 [42, 46]. Smad1 and Smad5 might also transmit TGF-β signals in some situations [42, 46]. The promiscuous Smad4 is shared by all Smads and is the only known co-Smad of the human genome [49]. Two vertebrate inhibitory Smads, Smad6 and Smad7 are inducible targets of the Smad pathway [46]. They provide a negative feedback signal for pathway regulation by transiently binding to the cytoplasmic domains of the type I receptors and preventing phosphorylation and activation of pathway-specific Smads [50-54].

Smads are structurally characterized as having an N-terminal MH1 (Mothers against decapentaplegic homology domain 1) and a C-terminal MH2 domain connected by a linker region [55-58]. The MH1 domain mediates DNA binding and contains the nuclear localization signal [46]. The MH2 domain, on the other hand, supports transactivation when fused to the Gal4-DNA binding domain and incorporates a structural feature known as the L3 loop, which is critical for the specific association of R-Smads and I-Smads to the L45 loop of type I receptors [59]. The L3 loop of the MH2 domain is also involved in homo- or hetero-oligomerization of Smads [60]. In the case of R-Smads, a conserved C-terminal SSXS motif, which is missing in I-Smads and Smad4, is a substrate of the protein kinase activity that resides in the cytoplasmic tail of the type I receptor [55-58]. Phosphorylation of $S^{*}XS^{*}$ relieves an inhibitory conformation to allow the L3 loop to engage in homo- or hetero-oligomerization for subsequent shuttling into the nucleus in association with Smad4 [60]. Additional phosphorylation sites in the linker region serve as substrates of protein kinases activated by other signaling pathways including MAPK and mediate cross-talk of the pathways [61-65]. In the nucleus, recruitment of activated Smad complexes to regulatory elements brings about activation or inhibition of target gene transcription through association with coactivators or corepressors, respectively [46]. An important difference between Smad2 and Smad3 is that a short insert in the MH1 domain of Smad2 interferes with the DNA-binding function of this domain [59, 66]. Thus, Smad2 assembly at DNA target sites occurs via interacting partners such as Smad4 or FoxH1/FAST1/2 [67-69]. Most studies suggest that Smad2 and Smad3 are interchangeable and show that they display considerable functional overlap and combinatorial actions [70]. It is clear from targeted inactivation studies, however, that Smad2 and Smad3 indeed control distinct developmental and cellular processes [70-74]. Smad functions are also subject to regulation by a variety of mechanisms that influence their stability, cellular distribution and phosphorylation status, discussions of which are beyond the scope of this review [75].

PITUITARY ACTIONS OF ACTIVIN AND ANTAGONISM BY INHIBIN

Activins (activin A or B) are assembled and secreted as disulfide-linked dimers of inhibin βA or βB subunits whereas inhibins are generated through heterodimeric association of the inhibin βA or βB subunits with the inhibin α subunit [23]. These TGF-β family members were initially characterized by their actions to stimulate (activins) or inhibit (inhibins) FSH secretion from the pituitary [23, 76-78]. The subunits, in particular βA and βB, are expressed in a wide variety of tissues and the corresponding dimeric forms act locally to regulate relevant and diverse biological processes [79]. Inhibins, more so than activins, have additional roles as classical endocrine factors [23] and substantial evidence implicates them in tumor suppression [80, 81]. In general, inhibins and activins are functional antagonists, but not all activin-responsive cell types display inhibin sensitivity consistent with the requirement for additional components. Several candidate proteins have been proposed to function as inhibin receptors or co-receptors [82-85]. The quest for an inhibin receptor or co-receptor led to the identification of betaglycan (or TβRIII) and the realization that this protein, previously characterized as a TGF-β2 co-receptor, indeed has a dual function to promote the actions of both TGF-β2 and inhibin [86].

The functional relevance of betaglycan in mediating inhibin antagonism is supported by a number of observations. Betaglycan is expressed in inhibin-responsive gonadotropes [87]. Betaglycan immunoreactivity at the membrane of gonadotropes and FSH levels show an inverse correlation in late estrus when inhibin levels are rising and establishing feedback control of FSH secretion [88]. When introduced into heterologous cells that otherwise do not display appreciable responses to inhibin, betaglycan expression at the cell surface is sufficient for high affinity ^{125}I-inhibin binding and high potency antagonism of activin by inhibin [86]. On the other hand, RNAi-mediated knockdown of endogenous betaglycan in primary cultures of rat anterior pituitary cells or in the LβT2 gonadotropic cell line compromises the ability of inhibin to antagonize activin-stimulated FSH secretion [89, 90]. Similarly, the inhibin-binding function of betaglycan is compromised in the presence of an immunoneutralizing antibody directed to an epitope of the extracellular domain of betaglycan that encompasses amino acid residues required for inhibin binding [89]. Whereas RNAi-mediated knockdown of betaglycan compromises its actions to promote TGF-β2 signaling and to mediate inhibin antagonism, the antibody selectively disrupts inhibin antagonism because a more distal portion of the extracellular domain seems to be sufficient for

high affinity TGF-β2 binding and function [89, 91]. A model of differential betaglycan-inhibin versus betaglycan-TGF-β2 action has emerged. Inhibin or TGF-β binding to betaglycan promotes high affinity interactions of these ligands with relevant type II receptors, ActRII, ActRIIB and BMPRII in the case of inhibin and TβRII in the case of TGF-β2, but leads to distinct outcomes for each ligand [91]. A ternary complex of betaglycan-TGF-β2-TβRII promotes TGF-β2 binding to the type I TGF-β receptor, ALK5, leading to increased cellular sensitivity to the ligand and activation of downstream Smad2/3 phosphorylation and signaling [92]. Inhibin binding to betaglycan also promotes high affinity binding of the ligand to type II receptors but in this case, the ternary complex of betaglycan-inhibin-type II receptor does not lead to type I receptor recruitment and, importantly, sequesters the type II receptors thereby preventing ligands such as activins or BMPs to assemble active ligand-receptor complexes and generate downstream signals [91, 93, 94]. The two functions of betaglycan are separable but competition between TGF-β2 and inhibin binding has been shown to affect inhibin potency in LβT2 cells [95]. Conversely, accelerated internalization of inhibin-bound betaglycan has been shown to antagonize TGF-β2 action [96].

Given the duality and complexity of betaglycan function, it might not be too surprising that betaglycan expression in a particular cell type is not predictive of inhibin sensitivity, suggesting that, in some cell types and under certain circumstances, additional components contribute to or interfere with the function of betaglycan as an inhibin co-receptor [97]. Pituitary lactotropes, for example, express endogenous betaglycan and are responsive to TGF-β but do not display measurable sensitivity to inhibin [87]. The mechanistic basis for this is not currently known but the observation is consistent with the notion that components other than betaglycan contribute to differential inhibin and TGF-β2 sensitivity. Betaglycan mutant mice display cardiac and liver defects and die during embryonic development probably due to deregulated TGF-β signaling [98]. As a result of the embryonic phenotype, this mouse model, while informative in as far as demonstrating the importance of betaglycan during embryonic development, has not been instructive in as far as delineating the *in vivo* function of betaglycan as a co-receptor for inhibin. Further evaluation of this question awaits the development of mouse models that permit targeted or even inducible deletion of betaglycan.

TGF-β ligands and receptors are present in the pituitaries of a variety of species including those of rodent, human and non-human primates. Studies of rodent anterior pituitaries show that inhibin α and βB subunit expression is restricted largely to gonadotropes [99] whereas inhibin βA is detectable in folliculostellate cells [100]. Primary pituitary cultures secrete activin B and activin A [101, 102]. Due to the low abundance of the secreted dimers, however, it has been difficult to quantify the local concentrations of dimeric activin or inhibin. Indirect evidence for the importance of local activin B in the rodent anterior pituitary ultimately came from *in vivo* and *in vitro* experiments in which an immunoneutralizing monoclonal antibody to activin B was utilized to show suppression of tonic FSH secretion and FSHβ mRNA expression without affecting LH levels [103-106]. As expected, the relevant type II receptors, ActRII and ActRIIB, are also present in the pituitary [107, 108]. ActRII is present throughout the pituitary, whereas ActRIIB expression is more limited to a subset of cells that includes gonadotropes [108].

Gonadotropes are the best characterized pituitary targets of activin but effects of exogenous activin A on most other pituitary cell types have been reported [109-113]. The primary action of activin on gonadotropes is to differentially activate FSHβ transcription and stimulate FSH secretion, but activin effects on LHβ expression have been reported [114]. The actions of activin are critical for the secondary FSH rise during the estrous cycle [115]. A variety of genetic models have been generated to assess the role of activin signaling in the pituitary. Despite the complications arising due to compensatory mechanisms, especially those that alter gonadal feedback signals, or broader defects that occur due to disruption of normal development and/or function of the gonads, these genetic models have provided a great deal of information [116]. For example, the importance of ActRII was revealed by studies of *Acvr2* mutant mice. These mice survived into adulthood but *Acvr2* loss of function led to female infertility, lower FSH levels, lower GnRH binding and compromised fertility of the males [117]. Genetic ablation of *Inhbb* was found to cause developmental anomalies as well as reproductive deficiencies in females but an unexpected rise in FSH levels presumably reflecting compensatory changes [118]. The loss of Smad3 was correlated with a reduced LHβ and FSHβ transcription [114, 119]. While these studies did not provide clear-cut answers, they confirmed that the activin system is important for optimal gonadotrope function and for maintaining a functionally intact reproductive axis.

Much of the current understanding of the mechanism underlying the actions of activin on the FSHβ promoter comes from studies of LβT2 cells as they express the relevant receptors that mediate activin and GnRH effects and the repertoire of required transcription factors [120]. The effects of activin on the FSHβ promoter are mediated largely through the Smad pathway, primarily Smad3 and Smad4, although the contribution of Smad-independent mechanisms has been implicated [121, 122]. Activin and GnRH act synergistically to activate the FSHβ promoter in LβT2 cells [123-125]. Indeed, synergism between activin and different patterns of hypothalamic GnRH pulses, in conjunction with peripheral feedback signals, are critical for promoting the differential pattern of LH and FSH secretion throughout the estrus cycle [126]. Activin seems to be pivotal for a variety of inputs that are known to influence FSH expression. Gonadal steroids, for example, can directly target the FSHβ promoter but also exert indirect effects on FSH expression by altering the local activin tone of the pituitary [127]. The mechanisms underlying gonadotrope-specific expression of key components including FSHβ, αGSU, LHβ as well as the GnRH receptor in response to activin, GnRH and a variety of other key regulators of this cell type have been the subject of numerous studies. Details of these mechanisms were recently summarized by two review articles and thus are not covered here [121, 122].

Activin stimulation of gonadotropes, in addition to initiating signals that are critical for FSH production, also generates feedback signals that either promote or terminate

further action. Activin has been shown to induce furin expression in pituitary cells and secondarily promote further processing of the activin subunit precursors and thereby increase ligand availability [128]. Activin induction of follistatin expression in pituitary gonadotropes, on the other hand, bio-neutralizes activin locally and leads to diminished signaling [129]. An additional action of activin to induce Smad7 imposes breaks on further Smad2/3 signaling and leads to diminished activation of downstream targets of the pathway [130]. Although many aspects of activin action on gonadotropes have been elucidated thus far, the extent to which observations from rodent models and cell lines can be extrapolated to the human pituitary requires further effort. Human pituitary adenomas express inhibin and activin subunits and the relevant signaling receptors and comparisons of normal pituitary tissue to adenomas suggest that, unlike the pattern seen in rodent pituitaries, ActRII rather than ActRIIB might be more relevant in the human pituitary [131-133]. The inhibin βB subunit is expressed at higher levels in FSH-producing pituitary adenomas compared to non-functioning adenomas, suggesting that this subunit is associated with gonadotropes, as it is in the rodent pituitary [132]. Cultured neoplastic cells derived from human pituitary adenomas display responses to activin and are growth-inhibited in response to this ligand [20, 134]. Interestingly, neoplastic pituitary cells also display diminished follistatin mRNA expression, suggesting that an imbalance in the activin and follistatin ratio might be a contributing factor [20].

ACTIONS AND MODULATION OF PITUITARY FOLLISTATIN

Follistatins are cysteine-rich monomeric glycoproteins that bind and bioneutralize activin with high-affinity at a molar follistatin to activin ratio of 2: 1 [135, 136] but also bind and modulate the actions of myostatin and several BMPs [137-140]. Recent structural studies have provided insight into the basis of the bio-neutralizing action of follistatin [141-145]. The follistatin: activin crystal structure has suggested that antagonism is achieved because both type I and type II binding sites on activin are masked when two follistatin molecules are bound to dimeric activin [143]. Mutagenesis of type I binding residues on activin A, however, have little effect on the affinity of activin for follistatin whereas most but not all Alanine substitutions in the type II binding interface substantially affect follistatin binding to activin, indicating that follistatin and type II binding requirements on activin are not identical [145]. Follistatins were first identified as FSH-suppressing gonadal factors but are now appreciated to have broad significance in embryonic and adult tissues [140, 146, 147]. Follistatin and its ligands are often co-expressed [140, 148, 149] and many of the demonstrated actions of follistatins are presumed to reflect their local autocrine/paracrine bioneutralizing actions. Deregulation of follistatin function is associated with a variety of cancers, including pituitary adenomas [20] and has been implicated in tumor metastasis [150, 151]. Genetic ablation of *Fst* causes many embryonic defects, including skeletal and cutaneous abnormalities, and causes mortality shortly after birth [152]. Follistatin overexpression, on the other hand, is associated with varying degrees of infertility, suppression of FSH levels only in one transgenic line that displayed widespread transgene expression as well as morphological defects leading to early arrest of folliculogenesis but variable effects on testis size [153]. Whereas the role of follistatin in spermatogenesis still remains an open question [154], the importance of this antagonist for the maintenance of folliculogenesis has received further support from anomalies seen in mice with granulosa-specific inactivation of *Fst* [155].

A single follistatin gene encodes two alternatively spliced mRNAs from which the FST-315 and the C-terminally truncated FST-288 variants are generated [156, 157]. An additional FST-303 form is a proteolytic cleavage product of FST-315 [140]. In general, the shorter FST-288 form is presumed to act locally due to its higher affinity for cell surface proteoglycans while FST-315 is the predominant circulating form [158]. The differential significance of these spliced forms remains largely unresolved. Data from genetic mouse models engineered to express only one spliced form of follistatin, however, are strengthening the notion that different isoforms have distinct functions [159, 160]. One of these studies reported that FST-315 but not FST-288 overexpression rescued the embryonic lethality of the *Fst* null mice but the mice displayed anomalies including female infertility resulting from ovarian defects, suggesting that some or most of the functions of FST-288 and FST-315 are not interchangeable [160]. The other study reached the conclusion that whereas FST-288 is sufficient for full survival of mice into adulthood, it is not sufficient for full fertility [159]. Despite the different conclusions reached, the results of these two studies clearly highlight the physiological relevance of isoform-specific actions of follistatin.

Several lines of evidence from *in vitro* and *in vivo* studies support the contribution of intra-pituitary follistatin to the control of reproductive functions. Follistatin transcripts are present in most anterior pituitary cell types, including gonadotropes and folliculostellate cells [161, 162]. The protein has been purified from bovine folliculostellate cells [163], bovine pituitary homogenates [164], ovine anterior pituitary cells [165] and shown to be secreted by rat anterior pituitary and folliculostellate (FS/D1h) cells [100, 166]. Perturbation of the local availability of activin in the presence of a follistatin-specific antiserum alters the potency of exogenous activin A to stimulate FSH secretion from cultured rat anterior pituitary cells [166]. A mutant form of activin A (K102E-activin A) that fails to bind to cell surface activin receptors but retains follistatin binding elevates basal FSH secretion by displacing activin from follistatin thereby increasing its local availability [141]. Antisense-mediated knockdown of endogenous follistatin leads to an increase in FSHβ mRNA [167]. Pituitary follistatin mRNA levels show dynamic fluctuations during the estrous cycle in a manner generally consistent with its role in modulating FSH production [168, 169] although the results of one study led to a different conclusion and suggested that the action of follistatin is to facilitate rather than suppress FSH production [170]. Pituitary follistatin levels are modulated *in vitro* by exogenous inhibin and by the feedback action of ovarian inhibin *in vivo* [171]. Gonadectomy elevates pituitary follistatin mRNA levels in both male and female rats, probably reflecting the loss of inhibin feedback from the gonads but also resulting from increased GnRH secretion

[172, 173]. The modulation of pituitary follistatin expression and changes in the intra-pituitary activin/follistatin balance has been proposed to, in part, indirectly mediate the actions of steroids on gonadotropin expression [174, 175]. Follistatin mRNA levels in the rodent pituitary are correlated with GnRH pulse frequency with rapid frequencies supporting maximal follistatin but no change in FSHβ mRNA and slower frequencies causing a selective rise in FSHβ but no change in follistatin mRNA [176].

GONADOTROPE-SPECIFIC ACTIVATION OF FOLLISTATIN BY ACTIVIN

Gonadotropes and folliculostellate cells are the primary sources of follistatin in the pituitary [99, 100]. Activin and GnRH exert direct effects on follistatin expression in gonadotropes and thereby activate an autocrine feedback loop that terminates further activin action [177, 178]. Folliculostellate cell-derived follistatin, on the other hand, exerts paracrine control of activin action on gonadotropes [179-181]. Activin is a potent inducer of follistatin mRNA expression in preparations of primary rat anterior pituitary cells [182]. This action of activin is antagonized by inhibin co-treatment, suggesting that gonadotropes are the primary source of activin-induced follistatin expression [182]. Consistent with this conclusion, *Fst* transcription is induced by activin treatment of αT3-1 and LβT2 cells, two gonadotrope-derived cell lines [183]. Furthermore and contrary to expectations, activin has no effect on follistatin expression or secretion from pituitary folliculostellate cells despite their sensitivity to other actions of activin [100]. Folliculostellate cells, however, respond to pro-inflammatory cytokines such as interleukin-1β with a substantial increase in follistatin production [100]. These observations have led to the identification of a novel mechanism for gonadotrope-specific induction of *Fst* in response to activin signaling. The promoters of the rat or human *FST* genes have been evaluated in a variety of cell lines and shown to be targets of inputs such as the cAMP/TPA pathway in P19 and F9 embryonic carcinoma cells [184, 185], the Wnt/β-catenin pathway in NCCIT human embryonic carcinoma and F9 teratocarcinoma cells [186, 187] and by PPARα and PPARγ nuclear receptors in LβT2 cells [188]. GnRH activates the *Fst* promoter in LβT2 cells [189-191]. In non-pituitary cells such as HepG2 or HEK293T cells, the *Fst* promoter is induced by activin or TGF-β via Smad2/3 [192, 193]. Unexpectedly, the same promoter fragments are unresponsive to activin in αT3-1 or LβT2 gonadotrope cells [183, 192]. The basis for this observation was resolved by the discovery that a Smad-binding element (SBE1) located in the first intron is required for activin-dependent induction of the *Fst* transcription in αT3-1 and LβT2 cells [183]. Smad3 but not Smad2 recruitment to SBE1 activates *Fst* transcription in response to activin [183]. Whereas SBE1 is absolutely required for activin/Smad3-dependent induction of *Fst* transcription in αT3-1 or LβT2 cells, it does not mediate activin effects in non-gonadotrope pituitary cells including FS/D1h and TtT/GF folliculostellate cells (unpublished observations). In non-pituitary cells, such as HepG2 or HEK293T, upstream regulatory elements mediate Smad2/3 effects on *Fst* transcription and SBE1 is dispensable [193-195].

The basis for gonadotrope-specific activation of the *Fst* gene via SBE1 was resolved through an unbiased proteomic screen for Smad3 partners. These efforts led to the identification of Forkhead box L2 (FoxL2), a forkhead transcription factor and an obligatory partner of Smad3 for SBE1-dependent activation of *Fst* transcription [193]. FoxL2 and Smad3 bind to two adjacent elements (FKHB and SBE1, respectively) located in the first intron of the *Fst* [193]. FoxL2 is permissive for Smad3-dependent activation of transcription and disruption of either element compromises activin-dependent activation of *Fst* in αT3-1 or LβT2 cells [193]. RNAi-mediated knockdown of endogenous FoxL2 in αT3-1 or LβT2 (unpublished observations) cells attenuates activin/Smad3-dependent induction of *Fst* transcription [193]. Smad3 binding to the SBE1 site is induced upon initiation of activin signaling whereas FoxL2 recruitment to FKHB is less responsive to activin treatment and a portion of cellular FoxL2 seems to be constitutively bound to FKHB [193]. As in the case of Smad2 and its obligatory partnership with FoxH1 for high affinity binding to cognate DNA sequences on certain target genes, it is likely that FoxL2 bound to the FKHB site stabilizes Smad3 binding to the SBE1 site and thereby promotes Smad3-dependent activation of the *Fst* in cells such as αT3-1 or LβT2 that express endogenous FoxL2 [193]. These observations raise the possibility that similar mechanisms of Smad2/3 partnerships with other forkhead proteins are also required for the modulation of follistatin expression in other cell types.

Interestingly, *Foxl2* (also designated P-FrK) is expressed during early stages of pituitary development in the embryo [196, 197]. Expression persists into adulthood, largely confined to *αGSU*-expressing gonadotropes and thyrotropes of the adult pituitary [193, 198]. This pattern suggests that FoxL2 plays a role during pituitary development as well as in differentiated gonadotropes and thyrotropes. FoxL2 actions in the developing pituitary are not known and a limited number of studies have only now begun to provide information about its relevance in the adult gonadotrope. *Gnrhr* transcription in αT3-1 cells is regulated through the functional interactions of FoxL2 with Smads and Ap-1 via a composite regulatory element [199]. Consistent with its pattern of FoxL2 expression in the adult pituitary, *Cga* was shown to be a target in both gonadotrope- and thyrotrope-derived cell lines [198]. FoxL2 is required for Smad3-dependent induction of *Fst* in αT3-1 and LβT2 cells [193]. Recently, FoxL2 was shown to also mediate Smad3-dependent induction of *Fshb* in LβT2 cells [200, 201]. Collectively, these data point to the possibility that, by modulating the expression of key targets of gonadotropes, FoxL2 plays an important role in the pituitary to regulate the reproductive axis. These findings have opened a new avenue of exploration of FoxL2 significance beyond its well-established role in the ovary [202-204].

The forkhead family is represented by a large number of transcription factors that exert diverse effects and participate in many morphogenetic and homeostatic processes [205, 206]. A highly conserved DNA-binding domain with a characteristic helix-turn-helix core motif defines this family, hence the "winged-helix" designation. FoxL2 is a divergent and evolutionarily conserved member of the family [207, 208]. Mutations in *FOXL2* are associated with a condition known as Blepherophimosis-Ptosis-Epicanthus Inversus

syndrome (BPES), with affected patients displaying eyelid defects and premature ovarian failure (POF) in female patients only with type I but not type II BPES [207, 209, 210]. FoxL2 is required for ovarian follicle formation and ablation of the gene disrupts granulosa cell differentiation, abnormal oocyte growth and infertility only in females [202, 203]. In the ovary, the transcriptional effects of FoxL2 on targets such as *Cyp19* [211, 212] and StAR [213] indicate a regulatory role in steroidogenesis. Inducible deletion of *Foxl2* in the adult ovary is sufficient to induce testicular markers in ovarian cells and reprogram them to testicular lineages, consistent with the requirement for FoxL2 to actively maintain ovarian follicles and prevent ovary to testis sex reversal [204]. The association of a missense mutation in *FOXL2* with adult type granulosa cell tumors has further implicated a role for this factor as a tumor suppressor [214]. Clearly, FOXL2 has well defined functions in the ovary. Further studies on the pituitary are needed to assess the importance of FoxL2 function in the pituitary. Recent studies of the pituitaries obtained from *Foxl2* mutant mice, however, are yielding compelling data. These experiments indicate that while loss of *Foxl2* does not alter the relative number of αGSU/LHβ-positive gonadotropes, it leads to a selective decrease in the number of gonadotropes that express FSHβ immunoreactivity ([215], manuscript in preparation).

SUMMARY

In summary, activin and its two antagonists, follistatin and inhibin, play crucial roles in the anterior pituitary to promote differential FSH production and thereby exert indirect control on folliculogenesis and fertility. Counter-regulatory mechanisms that keep activin in check might also be critical for preventing pathological conditions that give rise to tumors. Activin signaling in gonadotropes is subject to at least two such counter-regulatory mechanisms, including betaglycan-mediated inhibin antagonism of activin signaling and Smad3/FoxL2-mediated induction of *Fst*. A better understanding of the mechanisms and relative importance of these complex processes will not be trivial and would be facilitated through the development of novel tools, such as cell-type specific, inducible models that allow dynamic deletion or overexpression of key components that modulate them.

CONFLICT OF STATEMENT

LMB has nothing to report. WV is a co-founder, consultant, equity holder, member of the Board of Directors and Scientific Advisory Board of Acceleron Pharma, Inc. In accordance with Salk Institute policy, WV derives patent and licensing income in the activin field.

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