ABSTRACT

GORE, ARNOLD JESSE. Activin Induction of the Ovine Follicle-Stimulating Hormone β-Subunit is Mediated by Smad4 and a Forkhead Box Transcription Factor. (Under the direction of Dr. William L. Miller).

Follicle stimulating hormone (FSH) is a α/β glycoprotein produced in all vertebrate pituitary gonadotropes that is required for egg maturation, and enhances the performance and maturation of sperm. Currently, the precise physiological mechanisms that increase FSH are unknown, but are complex since its expression is controlled by at least 6 hormones. The most potent inducer of FSH is activin that directly stimulates transcription of its rate-limiting β-subunit (*FSHB*) by targeting specific elements within the promoter of the *FSHB* gene.

Activin is widely known to activate the transcription factors Smad2/3. It also activates mitogen-activated protein kinase (MAPK) pathways that regulate gene expression by phosphorylating various transcription factors. Early responses to Smad- and MAPK-mediated gene regulation typically occur rapidly and transiently (1-4 hrs). This rapid time-course does not explain progressive activin induction of an ovine *FSHB* (o*FSHB*) promoter/reporter construct in LβT2 cells, which peaks at 22 hrs. Therefore, induction of o*FSHB* by activin likely depends on secondary, or primary and secondary transcription factors.

Previously, a DNA sequence was discovered in our laboratory between -171 bp and -159 bp of the o*FSHB* promoter that is necessary for 68 % of activin induction in LβT2 cells, and is also required for 99.9 % of o*FSHB* expression *in vivo*. Thus, the activin-responsive proteins bound to this site are critical and physiologically important mediators of o*FSHB* synthesis and FSH production. This element consists of a Smad binding element (SBE) juxtaposed downstream to a putative Runx/forkhead box (FOX) protein binding site,

consistent with the necessity for secondary factors that are induced over time. This study sought to further characterize this region of the o*FSHB* promoter and the factors involved in activin induction of o*FSHB*.

Binding studies revealed that Smad4 from LβT2 cells binds a palindromic SBE at a maximal level after 20 hrs of activin, mimicking the progressive kinetics of o*FSHB* induction. This binding was competed 85 % with a wild-type o*FSHB* oligonucleotide that encompassed the -162 bp SBE, indicating that Smad4 can bind to the o*FSHB* promoter. Additionally, overexpression of a dominant negative Smad4 reduced the level of activin induction by 62 %. These data provide strong evidence that Smad4 is involved in activin-mediated o*FSHB* transcription.

Other studies ruled out participation of Smad2 and 3 in activin induction of o*FSHB*, but there is evidence supporting participation of a FOX transcription factor. Inhibition of p38, a kinase involved in o*FSHB* expression, blocked activin induction of o*FSHB* only after 8 hrs, dividing the progressive, activin-mediated increase into two phases. This suggested the involvement of an alternate and activin-regulated gene required for the second phase, such as *Runx* or *FOX* family members. Focus was directed towards FOX genes since *Runx* is not usually regulated. One candidate gene, *FOXQ1*, was identified that was transiently induced up to 4.5-fold by activin at 8 hrs. This correlated directly with the time at which the p38 inhibitor blocked o*FSHB* induction. Given that Smad4 is not activin-regulated, either transcriptionally or post-translationally, is not an independent transcription factor, and that the physiologically important site from -171 bp to -159 bp consists of FOX/Smad binding sites, FOXQ1 may be necessary for o*FSHB* synthesis. However, FOXQ1 is only one of 43 FOX family members all of which bind the same DNA sequences. Therefore, other FOX

proteins may also be important mediators of o*FSHB* induction. Further studies are required to prove that FOXQ1, and not another FOX member, is a key driver of FSH production.

Activin Induction of the Ovine Follicle-Stimulating Hormone β-Subunit is Mediated by Smad4 and a Forkhead Box Transcription Factor

by
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DEDICATION

To my mother and father for their constant love and support, and for teaching me the values, patience and diligence necessary for success. They have both been an inspiration to me. Also to my sister, a true friend who has kept life fun, and has always been there in times of need.

BIOGRAPHY

Jesse Gore was born in Richmond, VA to Arnold and Myong Gore on a late July morning in 1979. He grew up in Chesterfield, VA, a suburban area outside of Richmond where, during his childhood, he spent a lot of time exploring and enjoying the outdoors with various friends and his father, developing interests that have been retained to this day. It was here that he also participated in various sports, from baseball to competitive swimming, held jobs in both food service and retail, and learned to play the violin. After receiving his primary education in Chesterfield, he moved to Wilmington, NC to pursue his undergraduate studies at the University of North Carolina at Wilmington. Having a general interest in science he chose to major in Biology and minor in Chemistry. During his senior year, he obtained a National Science Foundation fellowship, under the direction of Dr. Stephen T. Kinsey that helped mold his interests in science, gave him exposure to basic research, and allowed him to work in the field collecting specimens. After graduating with a Bachelor's degree in the summer of 2003, he moved to Raleigh, NC and joined the laboratory of Dr. William L. Miller as a doctoral student in Molecular and Structural Biochemistry at North Carolina State University. In Dr. Miller's laboratory his scientific interests were further shaped by his exposure to the importance and complexity of biological systems. Now in the fall of 2008, Jesse looks forward to completing his graduate studies with a Ph.D. in Biochemistry, and pursuing a career of academic research and teaching.

ACKNOWLEDGEMENTS

I would first like to express my sincerest gratitude to Dr. William L. Miller, who has served as both an advisor and friend throughout my graduate studies. He has been an outstanding mentor to which I am most indebted, and I feel fortunate to have worked under his direction. He exposed me to every aspect of academic research, from laboratory techniques, teaching and writing, to the importance of thorough research, all of which have positively influenced my decision to pursue a career in academia. His exuberant personality and optimism have made it a pleasure to come to his laboratory every morning. I also thank Joyce Wu, Nedal Safwat, Pei Su and Farideh Shafiee-Kermani for welcoming me to the laboratory and getting me started, and our current laboratory members, Sang-oh Han and Jingjing Jia, for all of their assistance and keeping the lab a fun and interactive environment.

I thank my committee members for their advice and support over the years, with special thanks to Dr. Jun Ninomiya-Tsuji. Her advice has been beneficial to my research on numerous occasions. I also thank the graduate students of the Department of Molecular and Structural Biochemistry, many of whom have become close and certainly life-long friends, for making my time as a graduate student so enjoyable.

I thank my parents for their hard work to ensure that I was given every opportunity, for their endless encouragement, and giving me the independence to learn my own lessons in life. I thank my sister, other family and friends for their encouragement as well. I also extend my

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I. Follicle Stimulating Hormone: Structure and Function

Follicle Stimulating Hormone (FSH) is a pituitary hormone critical for the maintenance of normal reproductive function in all vertebrates. FSH is known as a gonadotropin because it affects only gonadal cells, and is necessary for their stimulation and gamete production. It is produced in the anterior pituitary solely within gonadotrope cells, which are named that because they are the only cells that produce FSH. In males, it acts only on Sertoli cells in the testes and enhances the maturation and performance of sperm (1). In females, it specifically targets granulosa cells in the ovary leading to the stimulation of follicle growth, or folliculogenesis (1), the process for which the name FSH is derived. Another pituitary gonadotropin, luteinizing hormone (LH), also acts on ovaries and testes, and in conjunction with FSH, plays a major role in the delicate mechanisms that regulate reproduction.

FSH is a heterodimeric protein composed of two subunits; α and β. Both subunits are post-translationally glycosylated on two specific asparagine residues, and tertiary structure is provided for the α- and β-subunits through five and six internal disulfide bonds, respectively (2, 3). These subunits are non-covalently joined to form a biologically active hormone that is secreted only by pituitary gonadotropes. Throughout the reproductive cycle, the circulating levels of mature FSH vary. Regarding folliculogenesis in humans, over the course of three cycles or months, increasing concentrations of FSH lead to the development of numerous follicles from immature primordial follicles to mature and significantly larger tertiary follicles that contain an ovulation-ready egg. More than 1000 follicles reach tertiary status

during the third month. Only a single, highly mature follicle will gain dominance and ovulate during the LH surge that is accompanied by minor FSH surge (4, 5). The other less mature follicles enter atresia (follicle apoptosis) due to a reduction of FSH levels (4). A secondary rise in FSH is thought to be important for recruiting follicles for the next ovulatory cycle. Therefore, the biological processes that mediate these changes in FSH levels leads to reproductive regulation.

Although the functional form of FSH exists as a heterodimer, it is important to note that the α-subunit is actually common to other pituitary hormones including LH and the thyrotrope-specific hormone, thyroid-stimulating hormone (TSH). Thus, the β-subunit confers specificity to each hormone and is rate-limiting for the overall production of these hormones. The physiological importance of the β-subunit of FSH, FSHB, was highlighted through the use of transgenic mouse models. Female mice deficient in FSHB lack estrous cycles and are sterile (1). Males, however, are fertile despite notable reductions in both sperm numbers and testicular size (1). This indicates that in males, FSH is not essential for spermatogenesis, but is for testicular volume and maximal sperm performance. These abnormalities can be rescued through pituitary-specific expression of a human *FSHB* transgene, indicating that the phenotypic effects were specifically caused by a deficiency in FSHB (6). Interestingly, massive overexpression of FSHB in mice also results in reproductive abnormalities and infertility (7). Controlled overexpression, however, causes increased ovulation (8) indicating that the physiological amount of FSHB is critical for maintaining normal reproduction. Since FSHB ultimately controls the biological functions of

FSH it is critical to understand the molecular mechanisms, both positive and negative that regulate *FSHB* and ultimately the levels of FSH *in vivo*.

II. Positive Regulation by Gonadotropin-Releasing Hormone

The decapeptide, Gonadotropin-Releasing Hormone (GnRH), is produced within the hypothalamus and acts on pituitary gonadotropes to stimulate synthesis and secretion of FSH. GnRH is released in a pulsatile fashion that can vary in both frequency and amplitude (9). While slower pulses tend to favor transcription of *FSHB*, faster pulses selectively induce expression of the β-subunit of LH, *LHB* (10). Precisely how GnRH can induce differential expression of *FSHB* and *LHB* is not completely understood, but certainly involves distinct regulatory mechanisms.

The GnRH receptor (GnRH-R) is a G-protein coupled receptor that upon stimulation by GnRH, induces multiple intracellular signaling pathways. Several components activated by these pathways have been documented to influence *FSHB* expression, such as cAMP, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and phosphatidylinositol-3 kinase (11-17, 20). Studies that focused on ovine *FSHB* (o*FSHB*) first indicated that PKC and AP-1 complexes were important for induction by GnRH (17, 18). Since MAPKs can be activated by PKC, these studies suggested possible interaction between various pathways activated by GnRH. Moreover, they suggested that MAPKs may be involved because they are known to modulate the activity of AP-1 complexes (19).

A subsequent study confirmed the involvement of PKC and MAPKs in the induction of o*FSHB* by GnRH, and in rodents, MAPKs seem to be important as well (13-15, 20). GnRH is capable of activating all MAPK members, including p38, extracellular signal-

regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), but precisely which MAPK regulates *FSHB* induction by GnRH seems to vary. For example, in rats ERK is involved in the response (13), however, in mice, p38 seems to be more critical even though a role of ERK was also implied (14). The involvement of JNK is less clear, but it has been suggested that it maintains basal *FSHB* expression in rats, although it does not play a role in the response to GnRH (15), even though it is GnRH-responsive. Collectively, these studies have shown that GnRH is capable of stimulating ovine and rodent *FSHB* production through MAPK pathways and AP-1 complexes. This is consistent with the fact that GnRH can induce a significant increase in *FSHB* expression within 2 hrs because transcription of most AP-1-responsive genes occurs rapidly (13, 19).

GnRH is responsible for 50 – 67 % of FSH levels *in vivo* (21), and hypogonadal (hpg) mice, which lack GnRH, are sterile due to low levels of LH and FSH (22). This genetic defect can be corrected to restore FSH production and fertility (22). Therefore, GnRH is undoubtedly important for FSH production, however, its inductive effects are rather weak and often indirect since GnRH stimulates general transcription and translation in gonadotropes. Throughout the species studied to date, GnRH only increases *fshb* levels by an average of 2- to 3-fold (13, 15, 17, 20, 23). The majority of FSH secretion actually seems to occur independently of GnRH receptor-mediated signals (24). Thus, other factors that positively regulate *FSHB* also merit attention.

III. The Transforming Growth Factor-β (TGFβ) Superfamily

Members of the transforming growth factor-β (TGFβ) superfamily have a diverse array of cellular functions including, growth, differentiation, and tissue reorganization. More

than 60 members have been identified thus far, with assignment to the superfamily being based on a seven-cysteine motif in a conserved C-terminal domain (25). These members include activins, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), TGFβs, mullerian inhibitory factor (MIF) and inhibins, and each member or group can contain multiple isoforms (Figure 1).

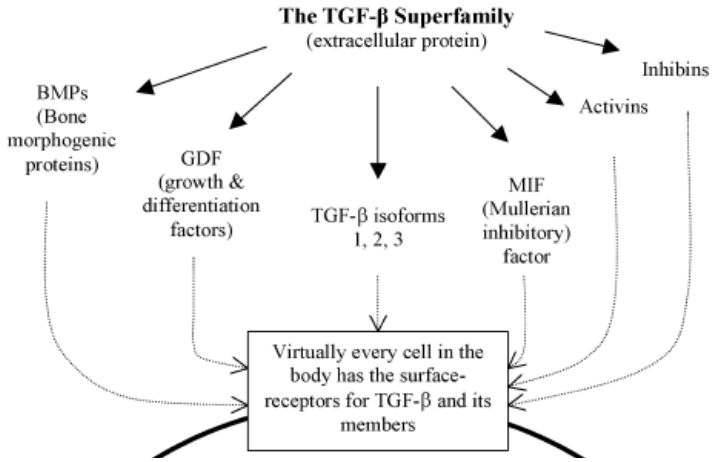

Figure 1: Members of the TGFβ superfamily (26). The TGFβ family consists of many members, such as BMPs, GDF, TGFβs, MIF, activins and inhibins. They can act on many different cell types within the body resulting in specific biological outcomes.

They are expressed within numerous cell types, but tend to be secreted near their targets, where they act as autocrine and/or paracrine factors to locally modify specific cellular functions in those cells with the appropriate receptors. These ligands initiate a series of complex intracellular signaling events that are necessary for maintaining normal physiological functions. Deregulated TGFβ family signaling has been implicated in many human diseases, such as autoimmune diseases, vascular disorders and cancer (27). Moreover, targeted disruption of some members is lethal in mice (28, 29). Some of the TGFβ

superfamily members, such as activins, have been found to elicit potent and direct effects on *FSHB* expression, either alone or in concert with other hormones like GnRH. They are believed to control the majority of FSH production *in vivo*, so their roles in *FSHB* regulation and thus, reproduction, deserve special consideration.

A. Activin

Activin was first isolated over 20 years ago from gonadal fluid as a nonsteroidal molecule and was named due to its ability to "activate" FSH secretion from the pituitary (30, 31). The induction of *FSHB* by activin is potent, with maximal expression being as much as 25- to 50-fold (13, 32, 33), and direct, since increases in *FSHB* primary transcript and mRNA levels are blocked by actinomycin-D (34). Like other TGFβ family members, activin is expressed in a variety of tissues, including the gonads and pituitary, which implies that *FSHB* regulation by activin may occur through endocrine, and paracrine or autocrine mechanisms. Due to the broad expression of activin, important roles in other bodily processes have also been discovered, such as in inflammatory responses (35), suppression of breast cancer cell growth (36), and erythropoiesis (37).

Like most members of the TGFβ family, activins are produced as precursor proteins, which are proteolytically cleaved to form active ligands. Structurally, functional activin exists as a homo- or heterodimer of two highly related β-subunits. There are three possible molecular species and their names reflect the type of subunit assembly: activin A (β_A-β_A), activin B (β_B-β_B), and activin AB (β_A-β_B) (Figure 2). Another dimeric TGFβ family member, inhibin, is composed of an activin β-subunit and a unique α-subunit to yield either inhibin A (α-β_A) or inhibin B (α-β_B) (Figure 2).

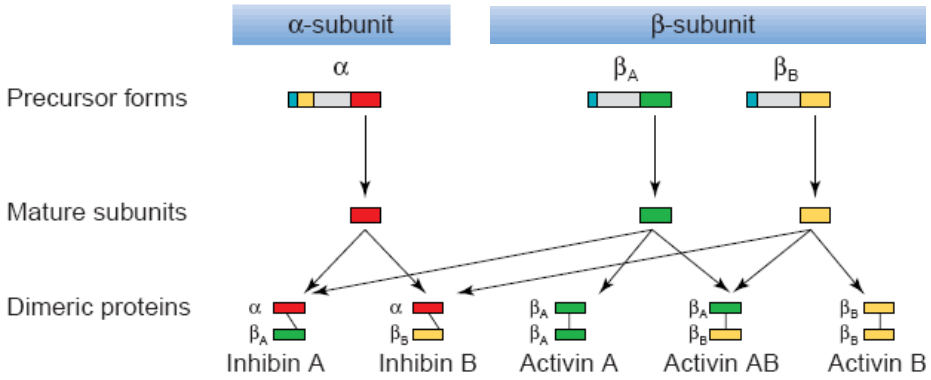

Figure 2: The assembly and various isoforms of activin and inhibin (38). Cleaved precursors produce mature forms of β_A (green) and β_B (yellow) subunits resulting in the production of three different activin homo- or hetero-dimers. Binding of either to a unique, mature α-subunit (red) yields two possible inhibin isoforms.

Both activin A and activin B can stimulate *FSHB* expression, although activin A seems to be the more potent of the two (39). Physiologically, however, activin B seems highly relevant because in rat pituitary cultures *FSHB* mRNA levels and FSH secretion is attenuated in the presence of a bioneutralizing antibody (40). An *in vivo* function of activin A on FSH production cannot be excluded, but its precise role is difficult to discern given that activin A deficient mice die at birth (28). Nevertheless, most *in vitro* studies of FSH regulation examine the actions of activin A since it is more potent and was the first commercially available isoform (41). Additionally, it binds to and activates the same receptors as activin B, initiating the same intracellular signaling pathways, thus making the substitution valid.

B. Receptors for the TGFβ Superfamily

The TGFβ family of ligands exert their biological effects through binding to two types of dimeric serine/threonine kinase receptors, termed type I and type II. These consist of a small extracellular domain and a large intracellular kinase domain. In vertebrates, there

are seven type I receptors, referred to as activin receptor-like kinases (ALK) 1-7, and five type II receptors. The type II receptor is constitutively phosphorylated and ligands bind them with high affinity (42), however, the signal is not transduced solely by type II receptor:ligand interaction. Instead, this complex recruits the lower affinity type I receptor, which cannot bind ligands on its own. Upon formation of the receptor:ligand complex, the type I receptor is transphosphorylated by the type II receptor in a region known as the GS box, which is critical for downstream signaling events. This generates the first step of an activin/TGFβ signaling pathway. The type I receptor then propagates the signal through a series of complex intracellular signaling pathways (Figure 3).

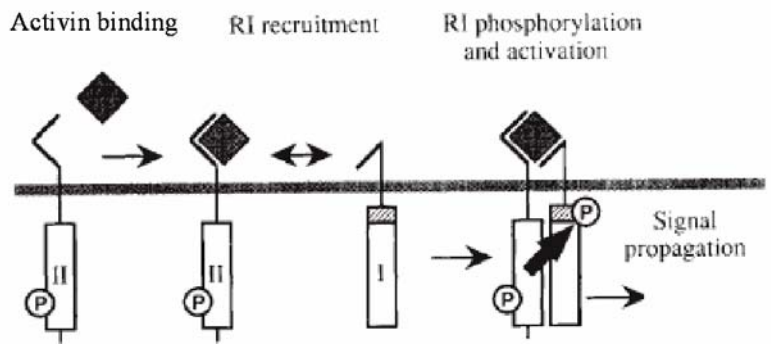

Figure 3: Model for signal initiation by the activin receptor (adapted from [42]). ActRII is the primary receptor that recruits type I receptors by means of a bound activin (diamond). Subsequent phosphorylation of the GS domain (striped box) by ActRII allows the type I receptors to propagate the signal to downstream substrates.

C. Activin Receptors and their Importance in FSH Regulation

For activin, there are two known type II receptors, ActRII and ActRIIB, and either is sufficient for interaction with and activation of type I receptors. The functional importance of the type II receptor alone was shown in ActRII deficient mice, which have reduced

fertility due to low FSH production (43). Two amino acids in activin, Asp-27 and Lys-102, are critical for type II receptor binding and biological activity. Substituting Ala for Lys at position 102 results in a 10-fold decrease in FSH release from rat pituitary cells (44).

Once bound to either of the type II receptors, the activin-ActRII/IIB complex can interact with four different type I receptors, ALK1/2/4/7, although, it only transduces signals through three, ALK2/4/7 (39, 45). To date, ALK2 is not known to modulate FSH production in response to activin, but both ALK4 and ALK7 are (39). For three reasons, ALK4 seems to be the preferred receptor-kinase that mediates *FSHB* expression. First, overexpression of ALK4, but not ALK7, significantly potentiates the ability of activin A to induce *FSHB* (39). Second, a constitutively active form of ALK4 is sufficient for induction (39, 46), and third, pharmacological inhibition of ALK4 blocks the ability of activin to induce FSH expression (47). The role of ALK7 is less clear, but may be the preferential receptor for transducing activin B signals (48). However, ALK4 may still be more important in activin B signaling since overexpression of a wild-type or kinase-dead form of ALK4 augments or inhibits *FSHB* induction by activin B, respectively, to greater extents than the same forms of ALK7 (39). Regardless of the activin:receptor complex composition, many intracellular signaling events are activated by the type I receptors. These can regulate transcriptional and thus, biological responses independently, or in conjunction with one another through cross-talk at various levels.

IV. Transduction of TGFβ Family Signaling Through Smad Proteins

A. Smad Classification and Structure

Perhaps the best characterized transducers of TGFβ family signaling are the Smad family of proteins that consists of eight members, known as Smads1-8. The Smad family is divided into three distinct classes: receptor-regulated (R-Smads), common-partner (Co-Smads) and inhibitory (I-Smads) (Figure 4). In vertebrates, there are five R-Smads (Smads1, 2, 3, 5, and 8), two I-Smads (Smads 6 and 7), and one Co-Smad (Smad4). Structurally, all Smads contain highly conserved sequences at their N- and C-termini, which are termed Mad homology (MH) 1 and MH2 domains, respectively, and are separated by a divergent proline-rich linker region (Figure 4). The N-terminal region of I-Smads, however, is only weakly similar to the MH1 domains of R- and Co-Smads, which may have a role in determining signal specificity (49). The MH2 domain of R-Smads is important for interaction with and activation by the type I receptors.

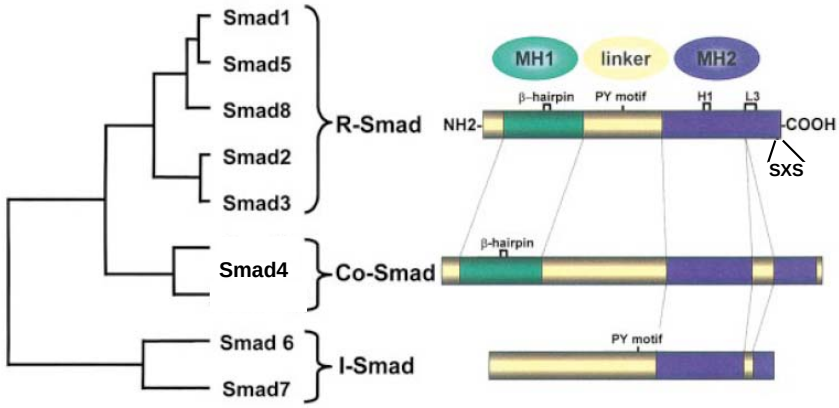

Figure 4: The Smad family (adapted from [27]). Phylogenetic tree and schematic structures of vertebrate Smads. There are three subgroups: R-Smads, I-Smads, and Co-Smads. MH1 (green) and MH2 (blue) domains contain sequences of high homology. The C-terminal SXS motif of R-Smads is phosphorylated by activated type I receptors.

B. Activation and Nuclear Translocation of Smads

Binding of R-Smads to an activated type I receptor results in direct receptor-mediated phosphorylation of the second and third serines in their C-terminal SSXS motif (Figure 4). Phosphorylation leads to conformational changes allowing R-Smad dissociation from the receptor, followed by homo- and hetero-meric complex formation that consists of two R-Smads and one Smad4 (50). The MH2 domain also mediates the formation of these Smad-Smad complexes. This active Smad complex then translocates into the nucleus where it can positively regulate transcription of target genes, such as Smad6 and 7, through direct contact of the MH1 domains to specific sequences termed Smad binding elements (SBEs) (Figure 5).

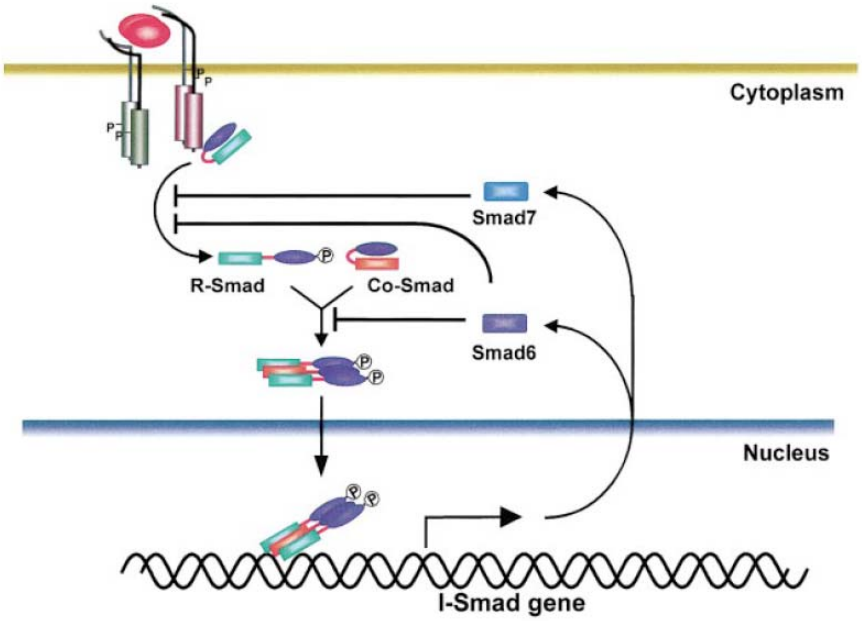

Figure 5: Activation of R- and Co-Smads and mechanism of action of I-Smads (adapted from [27]). Following activation of the type I receptor, R-Smads are phosphorylated. They dissociate from the receptor and form heteromeric complexes with Co-Smads. This complex translocates to the nucleus and regulates target gene (I-Smad) expression through direct binding to an SBE. The I-Smads negatively regulate signaling through prevention of R-Smad activation or R- and Co-Smad complex formation

Because Smad6 and 7 are inhibitory, their induction by TGFβ family members, such as activin, results in a negative feedback loop to control the intensity and duration of the signaling response (Figure 5). Signal cessation primarily occurs through their direct interaction with ALK4, preventing activation of all ALK4-mediated intracellular pathways. Alternative inhibitory actions for Smad6 and 7, such as prevention of R- and Co-Smad complex formation (Figure 5) and interference with Smad-DNA complex formation (51, 52), have also been reported.

C. Smad Binding Elements (SBEs)

Through DNA-binding site selection experiments, the nucleotides required for optimal Smad binding were identified as an 8 bp palindromic sequence (5'-GTCTAGAC-3') (53). Later, crystallography revealed that a single-copy or "half-site" of the palindrome, 5'-GTCT-3' or its complement 5'-AGAC-3', was sufficient for binding of a Smad 3 MH1 domain to DNA (54). Therefore, activated Smad complexes are thought to mediate transcriptional responses through direct binding to either single-copy or palindromic SBEs in the promoters of numerous target genes, including Smad6 and 7 (55, 56). It is important to note that unlike other R-Smads and Smad4, Smad2 cannot directly bind DNA. This is due to an additional 90 bp exon that alters the conformation required for binding within the MH1 domain. It is thought that homo- and hetero-meric complexes containing Smad 2 bind DNA through either Smad4 or Smad3 and 4, respectively (57). A naturally occurring Smad2 splice-variant that lacks the extra exon can bind DNA (58), thus like other R-Smads, this alternate form can regulate target genes through direct DNA interaction.

D. Receptor-Mediated Signal Specificity

The basic substrates of TGFβ family signaling (Smads) are quite similar, so various levels of specificity are required to manage cellular responses appropriately. The receptors alone are not sufficient for this since many of them activate the same R-Smads. One level of specificity occurs at the level of R-Smads. Although there are five members, only Smads 2 and 3 are activated in response to activin or TGFβ, and the other three respond only to BMP and GDF input, thus providing the first level of target gene selection. The Co-Smad, Smad 4, however, is not ligand restricted and can form complexes with all Smads. Because activins are the most potent and potentially primary inducers of *FSHB* synthesis, it is thought that following their activation, Smads2 and/or 3 may play major roles in controlling FSH production through direct binding to the *FSHB* promoter.

E. The Role of Smads in Activin Induction of *FSHB*

i. Mouse and Rat *FSHB* Expression

Studies focused on rodent *FSHB* promoters have, in fact, shown that both Smad2 and 3, as well as Smad4, substantially contribute to their induction by activin. Overexpression of Smad3 alone results in significant increases in both activin-independent and -dependent expression of mouse and rat *FSHB* (33, 59-61). Coexpression of Smad4 with Smad3 results in an even higher level of activin stimulation (59-61), and overexpression of both R-Smads (Smads 2 and 3) and Smad4 produces the highest increase in mouse *fshb* levels (59).

The importance of Smads 2 and 3 in activin induction of rodent *FSHB* was further substantiated through RNA interference (RNAi) technology. RNAi directed specifically towards either Smad2 or 3 reduces the ability of activin to induce rodent *FSHB* (33, 59, 62).

In addition, expression of a dominant negative Smad2, which has serine to alanine mutations in the C-terminal SSXS motif and thus cannot be activated by the type I receptor, interferes with *FSHB* synthesis within and secretion from mouse gonadotropes (63). Similarly, when a form of Smad3 that cannot bind DNA is expressed, the ability to potentiate activin-induced rat *FSHB* relative to wild-type Smad3 is lost (62). Collectively, these studies clearly suggested that activation of Smad2 and 3 were important for controlling *FSHB* production in rodents.

Examination of both mouse and rat *FSHB* promoters revealed the existence of a unique palindromic SBE, localized between -266 bp and -259 bp, that was likely to be critical for the transactivating actions of Smads 2, 3 and 4 (Figure 6). There are data that convincingly demonstrate that both Smad3 and 4 specifically bind this SBE following activin treatment. This is primarily shown with electrophoretic mobility shift assays (EMSA) that used specific antibodies to supershift protein:DNA complexes that contain Smad3 or 4, and mutant SBE probes that no longer retain binding ability (59, 61). Similar techniques have also implied that Smad2 can bind this SBE because specific antibodies compete for protein:SBE complex formation (59, 60), but do not definitively show it since the observed competition could be non-specific.

Mutation of this SBE also reduces the ability of activin to induce mouse *FSHB* by approximately 75 % (59). Two other and more highly conserved single copy SBEs further downstream have also been implicated as being important for activin induction of mouse *FSHB* (65). Currently, no evidence exists to suggest that Smads directly bind to either of these other two elements, although they may contribute slightly to Smad-mediated regulation

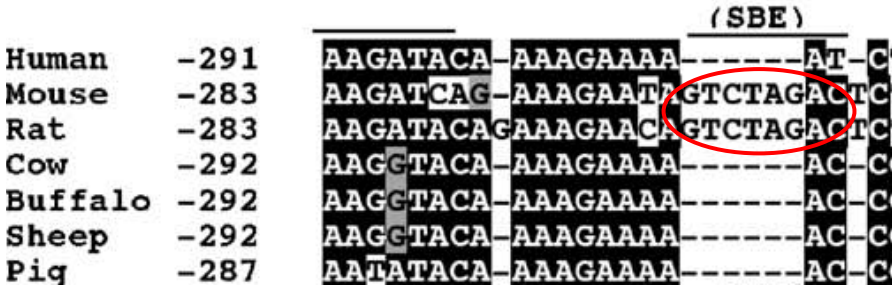

Figure 6: Sequence alignment of *FSHB* promoters (adapted from [64]). Alignment of sequences across species reveals the existence of a palindromic SBE that is unique to only mice and rats (red circle). All other species lack 8 bp within this region.

of rodent *FSHB*. Either of these two latter sites may account for only a mere 10 % of the strong activin-independent increases in *FSHB* observed by overexpression of Smad3 and 4 (61). Overall, these studies have shown that Smads 2, 3 and 4 significantly contribute to rodent *FSHB* regulation by activin primarily through their unique palindromic SBE. Further studies will be necessary to clarify the importance of the more proximal single copy SBEs.

ii. Ovine *FSHB* Expression

As mentioned above, only rodents harbor a palindromic SBE within their *FSHB* promoters (Figure 6). Mechanisms of activin regulation of *FSHB* in other species would thus seem more important to the ultimate goal of understanding what controls human *FSHB* synthesis and reproduction. Ovine *FSHB* (o*FSHB*) has therefore been used in our laboratory as a model to better understand human regulation since sequences between the two are more highly conserved, and because transgenic mice can be made to study o*FSHB* regulation *in vivo*.

Throughout the o*FSHB* promoter seven putative single-copy SBEs have been identified, but only two of them are reported to be important for mediating activin induction

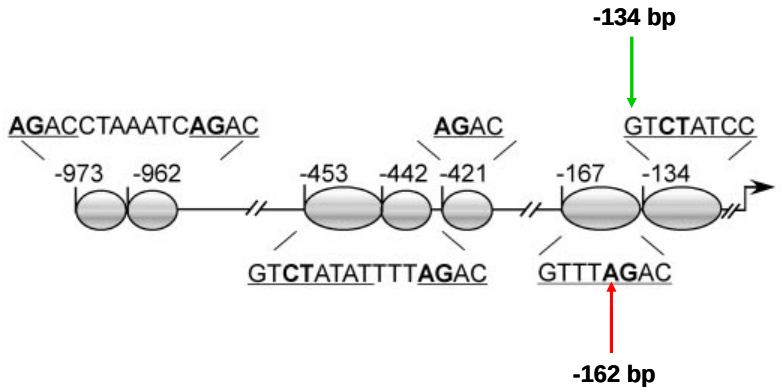

Figure 7: Putative SBEs within the ovine *FSHB* promoter (adapted from [65]). Analysis of the o*FSHB* promoter revealed the existence of seven putative single copy SBEs. Only two of these, one at -162 bp (red arrow) and one at -134 bp (green arrow) are important for induction by activin.

(Figure 7). Their starting positions are located at -162 bp and -134 bp and correspond to the two SBEs that are minimal in conferring Smad responsiveness to the mouse promoter as described above. Mutation of either site in the ovine promoter significantly reduces activin induction (65, 66). Although these SBEs were identified as important to activin action, the -162 bp site has not been shown to bind Smads. One study used EMSAs to suggest that Smad4 binds the more proximal -134 site because a Smad4 specific antibody competed for a small portion of the protein:DNA complex (65). This may have been non-specific competition however, particularly since there was no use of a negative control antibody. Therefore, in sheep and probably humans, Smads alone may not be as important for inducing *FSHB* in response to activin as they are in rodents.

Our laboratory further probed the potential involvement of Smads. Through the use of a dominant negative Smad3, Smad3(3SA), we observed that activation of Smad3 is not required for induction of o*FSHB* by activin (46). The Smad3 dominant negative has three serine-to-alanine mutations within the C-terminal SSXS motif, preventing its

phosphorylation and activation. This mutant Smad3 cannot block activin's ability to induce o*FSHB*, but can completely abrogate induction of a Smad-dependent gene that contains a palindromic SBE (46). This suggested that Smad3 activation by receptor-mediated C-terminal phosphorylation was not required for o*FSHB* induction. Smads 2, 3 or 4, however, may still be involved in activin induction of o*FSHB* because mutation of both putative single-copy SBEs prevents activin induction (65, 66).

There are also other reasons that Smads 2, 3, or 4 may be involved in o*FSHB* induction. First, Smad3 and 4 complexes might bind to either of the two SBEs independent of activin. In this case, they would be necessary for *FSHB* induction, but their activation through receptor-mediated C-terminal phosphorylation would not. Consistent with this, the divergent linker regions of Smad3 and 4 alone are known to be sufficient for ligand-independent transcriptional activation, when fused to a GAL4 DNA- binding domain (67, 68). Second, it is possible that Smad2 and 4 homomeric complexes may be more important for o*FSHB* induction because, as of 2005, we had only examined the involvement of Smad3. Lastly, alternative phosphorylation sites, such as within the linker region of either Smad2 or 3 may exist (see below, Section VII.A). Deletion of Smad3 and 4 linker regions results in a loss of transactivation of some genes, even though the MH2 domains can still be C-terminally phosphorylated by type I receptor and oligomerize (67, 68). This would suggest the possibility of an activin-dependent event that would not have been blocked by the C-terminal Smad3 mutant. This is difficult to examine experimentally because many of these sites are unidentified. Further characterization of these SBEs within the o*FSHB* promoter, as

well as the precise roles of either Smad2, Smad3 and/or Smad4, and their dependency on activin, will be crucial to unraveling the mechanisms that control reproduction.

F. The Importance of Co-Regulators in Mediating Smad Responses

The single-copy SBE is a relatively simple sequence of nucleotides, estimated to be present about once every 1024 bp in the genome (69). If this element were sufficient for Smad-mediated transcriptional responses, then massive numbers of genes would be non-specifically regulated in response to activin, as well as other TGFβ superfamily ligands. However, this is not the case. For example, in mouse gonadotropes activin induces or represses only 39 or 31 genes, respectively, by 2-fold or more after 48 hrs (70). Clearly, distinct mechanisms must exist to confer specificity to particular SBEs within one promoter or different promoters within the same cell, thereby allowing for target gene selection and another level of specificity.

Because the binding affinity of the Smad MH1 domain for the SBE is in the range of 10^{-7} M (54), which is too weak for effective binding *in vivo*, additional DNA contacts are generally required to achieve transcriptional activation. Smads can bind to palindromic SBEs independent of other proteins, but even then, SBE concateners are required to achieve Smad activation of a target gene (53, 71). Thus, interaction of Smads with nuclear partner proteins is the primary mechanism that controls the specificity of activin responsive genes. These cell-specific partners target Smad transcription complexes to promoters through high affinity binding sites, typically adjacent to SBEs (Figure 8). They also determine what other general transcription machinery is recruited, and how long the transcriptional response will

last (69). Therefore, regulation of *FSHB* by activin is likely to be strongly dependent on Smad-interacting transcription factors that target specific sequences adjacent to SBEs.

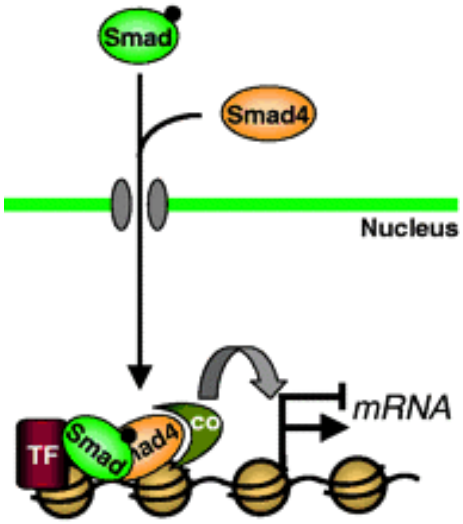

Figure 8: Smad partner protein interaction (adapted from [72]). Following R-Smad activation and binding to Smad4, the complex translocates to the nucleus and interacts with sequence-specific transcription factors (TF). Their binding sites are usually in close proximity to a SBE and the specific TF will determine co-activator and -repressor (co) recruitment to activate or inhibit gene expression, respectively.

i. Smad Co-Activators

The first transcription factor partner identified to interact with Smads was FAST-1 (FoxH1), a member of the winged-helix or forkhead box family of proteins (73). FoxH1 recruits Smad2/4 complexes to an activin responsive element (ARE) within target genes, such as *Mix.2* and *goosecoid*, through direct contact with Smad2 and stabilization by Smad4 (74). Since this discovery, Smads have also been seen to interact with at least 35 superfamilies of transcription factors, some of which contain numerous proteins (75). Positive, cell-specific regulation can occur through interaction with factors such as forkhead, Runx/AML (76), Pitx (77, 78), AP-1 (79), ATF (80), and SP1 (81) family members. Some

of these proteins are regarded as only DNA-binding adaptors for Smads since they lack a transactivation domain, while others are "stand alone" or bona-fide transcription factors that recruit Smads to certain promoters through sequence-specific DNA contacts. These could be constitutive, like some Runx members, or signal-activated like AP-1. Regardless of their precise assembly, Smad and partner proteins are required to target and induce specific genes through recruitment of the co-activators p300 and CBP. These generic co-activators are large proteins with separate regions for interaction with various transcription factors that do not contact DNA. They also have histone acetyl transferase (HAT) activity, suggesting that their recruitment may increase transcription through chromatin remodeling (69).

ii. Smad Co-Repressors

Smad interaction with proteins such as TGFβ-induced factor (TGIF), which also bind to specific DNA sequences, leads to histone deacetylase (HDAC) recruitment and subsequent repression of target genes. Two other homologous proteins, c-Ski and SnoN, are also repressors of activin signaling. They directly bind to Smads, but negatively regulate transcription in different fashions. They can compete with Smad and partner protein complexes for SBE binding (82), but can also bind Smads to either block their interactions with p300 and CBP (Figure 9A), or recruit HDACs leading to repressive chromatin modifications (Figure 9B). Unlike TGIF, their interaction with Smads is observed under basal conditions and disappears rapidly after ligand stimulation (85). Interestingly, both c-Ski and SnoN are transcriptionally regulated by TGFβ family members, so they can function to either protect against activation by Smad complexes in the basal state, or terminate the signal several hours later. Although there is currently nothing to suggest that c-Ski , SnoN or

other transcriptional repressors play a role in *FSHB* regulation by activin, there is evidence for the involvement of Smad-associated co-activators (see below).

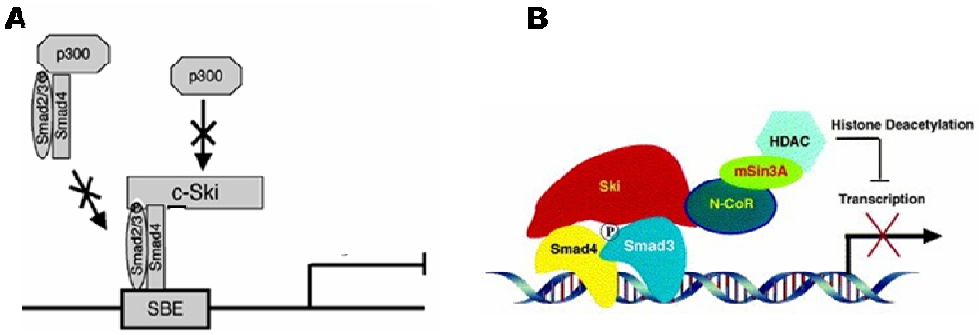

Figure 9: Mechanisms of c-Ski mediated inhibition of activin signaling. A, c-Ski binds to Smad complexes on SBEs and prevents their interaction with other p300-associated Smad complexes, thus preventing transcriptional activation (adapted from [83]). B, c-Ski interaction with Smads results in recruitment of general co-repressors and HDAC, which represses transcription through chromatin modification (adapted from [84]).

G. Involvement of Smad-Associated Proteins in Rodent *FSHB* Transcription

In the rat *FSHB* promoter, a putative Pitx binding element was discovered approximately 30 bp downstream of the palindromic SBE. This site specifically binds one member of the Pitx family, Pitx2c (60). The role of Pitx2c in activin induction may rely on its ability to interact with Smads because when this site is deleted, or Pitx2c is eliminated by specific siRNA, the ability of overexpressed Smad3 to induce a ligand-independent increase in *fshb* is lost (60, 77). In the mouse *FSHB* promoter, a more proximal Pitx binding site between -53 bp and -48 bp seems to be important (78). Mutation of this site also prevents ligand-independent induction by Smad2, 3 and 4 overexpression. Pitx2c, as well as Pitx1, directly bind Smad3 (77, 78), so cooperation between them may be critical for expression of both rat and mouse *FSHB*. The importance of Pitx proteins and their interactions with Smads

in o*FSHB* expression remains to be shown, but Pitx proteins may play a role since the proximal Pitx binding site is 100 % conserved between rodents and sheep.

H. Evidence for Smad-Associated Proteins in Ovine *FSHB* Transcription

Our laboratory has generated strong evidence for the importance of a transcription factor other than Smads in mediating activin induction of o*FSHB* that may itself be Smad-associated. The first indication was due to different activation kinetics between o*FSHB* and a Smad-dependent gene, p3TPluc, in response to activin. Induction of p3TPluc occurs rapidly and transiently (2-8 hrs), whereas for o*FSHB* it occurs more slowly and progressively over a 24 hr period (46). Because Smad2 and 3 activation also occurs rapidly (33), these kinetic studies suggested that neither R-Smad was likely to be the direct trigger for o*FSHB* induction, but they were for the Smad-dependent gene. The slow kinetics also suggested that o*FSHB* synthesis may depend on induction of one or more early and/or late response genes whose products may be Smad-associated proteins necessary to obtain the maximal level of *FSHB* transcription. This theory is consistent with the fact that many activin target genes are themselves Smad-interacting factors that generate "self-enabling" transcriptional responses (86). Such responses provide a mechanism for maintaining transcriptional responses over extended periods.

Other, and perhaps stronger, justification was through the identification of a site juxtaposed upstream of the more distal, single-copy SBE at -162 bp. When mutated, activin induction is lost, and *in vivo*, the mutation decreases o*FSHB* expression by 99.9 %, showing that physiologically, these sequences are extremely important for activin action. This sequence was initially identified by computer analysis to be a putative Runx1 binding site,

which was encouraging because Runx family members (Runx1-3) are known to interact directly with Smads (87, 88). Interestingly, Runx members bind to sequences homologous to those for forkhead, known as forkhead-box (FOX), proteins. These too are also known to interact directly with Smads (75, 88, 89). Therefore, either Runx or FOX proteins may act through this site in conjunction with Smads to induce o*FSHB* in response to activin.

Complexes that contain Smads are important for the more proximal SBE at -134 bp of the o*FSHB* promoter as well. This site, which is required for maximum levels of activin induction, binds Smad4, and the TALE homeodomain proteins, Pbx1 and Prep1 (65). The three proteins bind directly to each other, and Pbx1 and Prep1 also bind to both R-Smads (65). This multi-meric complex, however, binds under basal conditions and no changes are observed in response to activin. Therefore, these novel Smad partners may be important only for basal expression of o*FSHB*, and not for its induction by activin. The physiological importance of this site remains untested, so characterization of the site adjacent to the SBE at -162 bp will be critical. Although difficult because the Runx and FOX families contain 3 and 43 members, respectively, identification of this biologically important partner protein will be necessary to unlock exactly what drives activin-induced ovine, and possibly human, *FSHB* transcription since Smad3 activation alone is not sufficient.

V. Signaling Through Mitogen-Activated Protein Kinases (MAPKs)

In addition to Smads, TGFβ family members also initiate other signaling events, including MAPK cascades. All three MAPKs, ERK, JNK, and p38, are activated in response to receptor stimulation (90), which occurs through a series of phosphorylation steps. First, receptor activation results in phosphorylation of a MAPK kinase kinase (MKKK), such as

TGFβ-activated kinase 1 (TAK1) or MEKK1. These then phosphorylate various MAPK kinases (MKK or MEK) that are specific in their activation of the three different MAPKs. MKK3 and 6 activate p38, MKK4 and 7 activate JNK, and ERK is activated by MEK1 or 2. The MAPKs then transduce the signal through phosphorylation of various transcription factors that selectively alter transcription of target genes. For example, JNK- and p38-mediated phosphorylation of c-Jun and ATF2 results in activation of AP-1 and CREB target genes, respectively (91). In many cases, target gene activation is rapid, indicating a direct transcriptional response. Sometimes, however, their kinetics are slower, implying a delayed or indirect effect. Many rapid responders, known as immediate early genes (IEGs), are transcription factors on their own, such as c-Jun, that can be important for mediating transcription of late-responding genes. The variation in the specific response depends largely on the magnitude and duration of the signal, as well as the cellular conditions (92).

A. Smad-Independent Transcriptional Responses

The first indication that the TGFβ family could activate some genes independent of classical Smad-mediated transcription was through the use of Smad4-deficient cells and dominant negative Smads, which maintain transcriptional responses to TGFβ treatment (93). In addition, a mutant TGFβ type I receptor, defective in Smad activation, retains the ability to activate both JNK and p38 pathways (94). In other systems, JNK and ERK are critical for TGFβ-mediated inhibition of vascular endothelial cell migration, and p38 is necessary for activin to inhibit growth of breast cancer cells (95, 96). The kinase p38 is also required for activin-mediated inhibition of *Pit-1*, a pituitary transcription factor (97). Each of these studies, as well as some others, have defined the importance of MAPKs in mediating activin

and TGFβ responses without a need for Smads. However, many of the mechanisms, as well as their biological consequences are poorly characterized.

B. The Role of MAPKs in *FSHB* Regulation by Activin

In mouse gonadotropes and primary sheep pituitary cultures, both ERK and p38 are rapidly phosphorylated in response to activin (63), so it is likely that one or both of these MAPKs may be involved in *FSHB* induction. Importantly, our laboratory characterized the MAPK pathway in regulating o*FSHB* in detail. The MAPKKK, TAK1, is critical for induction of o*FSHB* by activin. Inhibiting this kinase pharmacologically completely abrogates activin induction of o*FSHB*, and overexpression of a catalytically inactive form of TAK1 significantly reduces o*FSHB* induction (46). TAK1 seemed to mediate its effects through p38 since inhibition of this kinase also reduced the level of activin-induced o*FSHB* expression. Inhibition of TAK1 also blocks activin-induced mouse *FSHB* expression by 99 % (46). A subsequent study indicated that p38 is also important for mouse *FSHB* induction (14). Therefore, TAK1-mediated activation of p38 seems necessary for activin induction of both mouse and ovine *FSHB*. This likely occurs through either MKK3 or 6, but neither have been studied with respect to *FSHB* regulation. The other two MAPKs, JNK and ERK, do not seem to mediate any of activin's actions on *FSHB* (13, 56, 61). It will be important to investigate p38 and its contribution to *FSHB* expression further.

VI. Intracellular Cross-Talk Between Smad and MAPK Pathways

A. Post-Translational Smad Modification

Although both Smad and MAPK pathways can function independently of one another to regulate genes, cross-talk exists that furthers the complexity, as well as the specificity of

TGFβ family signaling. One major point of convergence is through direct post-translational modification of Smads by MAPK members (Figure 10). Although receptor-mediated C-terminal phosphorylation is generally a critical step in TGFβ and activin signaling, recent data suggest that MAPKs can phosphorylate other sites, primarily within the linker or N-terminal regions of Smad 2 and 3 (Figure 10). This can occur by all three MAPK members on serine and threonine residues thereby modulating Smad activity and the resulting transcriptional responses.

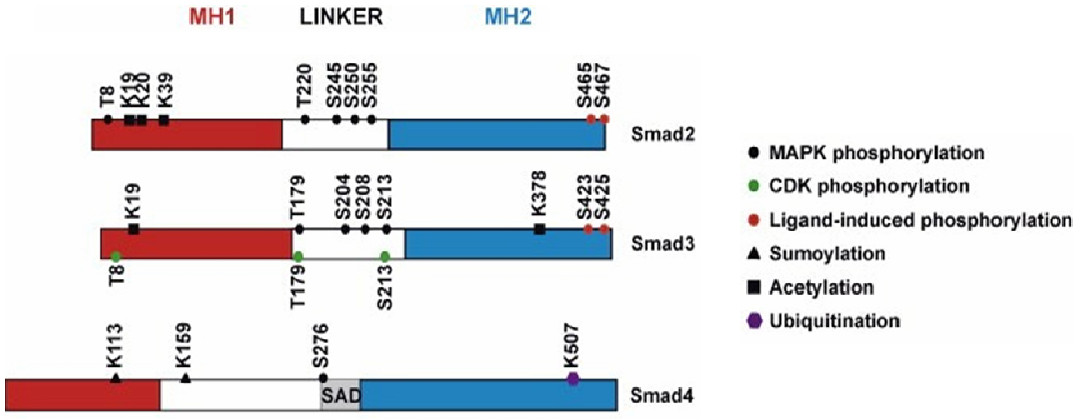

Figure 10: Post-translational modifications of Smads 2, 3 and 4 (adapted from [75]). In addition to C-terminal ligand induced phosphorylation, Smads2 and 3, as well as Smad 4 can be post-translationally modified on other residues within the MH1, linker, and MH2 domains in response to activin and TGFβ signals. These modifications can alter the functions of Smads in both positive and negative fashions.

Activin-mediated activation of the MAPKKK, MEKK1, an upstream activator of ERK, JNK and p38, can stimulate linker phosphorylation of Smad2 (98). Similarly, JNK has been reported to phosphorylate the linker region of Smad3 (93). Both of these events enhance transcriptional activity due to nuclear Smad stabilization, and stimulation of heteromerization with Smad4 and interactions with co-activators such as p300 and CBP.

Conversely, ERK-mediated linker phosphorylation of Smads2 and 3 prevents nuclear translocation and inhibits transcription (99). ERK phosphorylation of Thr 8 in the MH1 domain of Smad2, however, enhances transcription of an activin responsive gene (100). Surprisingly, ERK can phosphorylate Smad4, even though Smad4 is generally thought of as un-modified. This too is important for ligand-induced nuclear accumulation and transcriptional responses that involve Smad4 (101).

Smad stability and function is also regulated by other post-translational modifications. These include acetylation, ubiquitination and sumoylation, as well as phosphorylation by other kinases, such as cyclin-dependent kinases (Figure 10). Interestingly, another Smad4 modification was recently discovered. Sumoylation of Lys 159 by SUMO-1 is enhanced by the p38 MAPK pathway, and this modification is important for TGFβ-dependent transcription (102). Collectively, the various modifications elicit both positive and negative effects to modulate TGFβ and activin transcriptional responses. Alternate Smad phosphorylation or sumoylation sites have not been examined in *FSHB* regulation by activin, but would be interesting to pursue given that MAPKs are involved, and activation of Smad3 by receptor-mediated C-terminal phosphorylation is not important for o*FSHB* expression (46), as described above (see section V.Eii).

B. Cooperativity in Transcriptional Regulation

Another level of cross-talk between Smad and MAPK pathways is through the requirement of both for transcriptional responses. This generally occurs in the absence of Smad modification by MAPKs, and often, specific transcription factors serve as the common targets to directly regulate gene expression. For example, following ligand addition, TAK1

activates p38 through MKK6 resulting in phosphorylation of activating transcription factor-2 (ATF-2). ATF2 then binds to nuclear Smad3 and 4 to form an active complex that binds to cAMP response elements (CREs) and induces transcription (103, 104). Expression of a non-phosphorylatable form of ATF2 can prevent TGFβ-induced gene expression (104), showing that it is a common nuclear target of both Smad and MAPK pathways. Similarly, both JNK-activated AP-1 complexes and Smads are required for TGFβ/activin induction of the *Smad7* gene in rat kidney fibroblasts (105). The induction is lost when either an AP-1 site or SBE is mutated, indicating that both pathways are important, even though no physical interaction between Smads and AP-1 was observed in these cells.

A particularly interesting example of Smad- and MAPK-mediated gene expression is the induction of *Runx2* by TGFβ family members. This occurs very rapidly since *Runx2* mRNA levels peak within 1 - 2 hrs and fade shortly thereafter (106), consistent with direct activation by both Smad and MAPK pathways. Runx2 then activates various target genes possibly in conjunction with Smads (89). A similar mechanism may exist in which o*FSHB* is induced in response to activin since its kinetics are slow (46). In this case, neither Smad nor MAPK would directly activate o*FSHB*, but both would be important for the induction of IEGs, such as Runx or FOX members. Because continuous receptor stimulation maintains Smads in the nucleus (107), they could partner with the IEG product and bind to the physiologically important site in the o*FSHB* promoter described previously (see section V.H). The Smad-associated factor important for o*FSHB* induction may be directly regulated by activin in either Smad- and/or MAPK-dependent fashions.

VII. Negative Regulation of FSH Production

As described in detail above, FSH production is positively regulated by both GnRH and activin, but the most potent and well characterized of these inducers is activin. However, the mechanisms that negatively regulate *FSHB* are also important since the levels of FSH rise and fall throughout the reproductive cycle. The major negative regulators function by blocking the effects of activin.

A. Inhibin

Inhibin is a gonadal peptide originally isolated from ovarian follicular fluid (108). Two types of dimeric isoforms exist and are composed of a unique α-subunit and different β-subunit shared by members of the activin (activin A or B) family (see section IV.A and Figure 2). It is predominantly produced in Sertoli and granulosa cells in males and females, respectively (109), but subunit expression has also been localized to other tissues, including the anterior pituitary (110). Inhibin functions as an endocrine feedback regulator that inhibits FSH production. One study that aided in defining the physiological importance of inhibin showed increased plasma FSH and pituitary *FSHB* mRNA levels in the presence of a bioneutralizing antibody against the α-subunit (111). Conversely, injection of recombinant inhibin A produces the opposite effects (111). The mechanism of how this occurs is through antagonism of activin signaling. Inhibin, through its β-subunit, binds to ActRII or ActRIIB (112, 113). Its affinity for the type II receptors, however, is 10-fold less than activin (112), so a co-receptor, betaglycan, is required for higher affinity association (114). This strong binding sequesters type II receptors from binding activin and recruiting type I receptors, such as ALK4, thus preventing activin-induced *FSHB* transcription and FSH secretion.

B. Follistatin

Follistatin, structurally unrelated to inhibin and activin, is another negative regulator of FSH. It is produced in a variety of tissues including most cell types of the anterior pituitary (115, 116). Gonadotropes themselves are responsible for a substantial amount of pituitary follistatin, but the primary source is thought to be from pituitary folliculostellate cells. This provides both autocrine and paracrine mechanisms to locally control the level of *FSHB* production. Feedback regulation from other tissues is also possible.

Follistatin exists in two forms, follistatin-288 and -315 (FS288 and FS315), which are generated by alternative splicing. FS315 is the predominant form, but FS288 suppresses FSH 8- to 10-fold more than FS315 (117). Specifically, it functions by directly binding activin, masking the binding sites on activin for type I and II receptors (118). Activin is therefore biologically inactive and incapable of inducing *FSHB*. Both follistatin and inhibin, in conjunction with positive regulators, such as activin, create the delicate balance necessary to achieve physiologically relevant levels and patterns of FSH production, and maintain normal reproductive processes.

VIII. Models for Studying Transcriptional Regulation of *FSHB*

Many early studies that focused on FSH production were performed in primary pituitary cell cultures, which made it difficult to examine *FSHB* expression at the transcriptional level. The primary disadvantage in using primary cultures is that they are heterogeneous. Only 5 - 10 % of these cultures are gonadotropes, making it difficult to distinguish cell-specific responses. The other, more abundant cell types could also influence FSH production from gonadotropes through paracrine mechanisms. This can blunt or

enhance the magnitude of the response making data, such as the level of activin induction, difficult to interpret. Moreover, it is impossible to study gonadotrope-specific molecular mechanisms, such as Smad phosphorylation and transcription factor:DNA complexes, in mixed pituitary cell cultures following ligand addition since many cell types express activin receptors, and activin can activate Smads and other pathways in all of them.

Transfections with *FSHB* reporter constructs into heterologous cells provided preliminary methods to specifically study transcriptional regulation of *FSHB* (17, 18). However, this has disadvantages as well because often, heterologous cells lack distinct transcription factors, kinases and receptors necessary for cell-specific responses. For example, no cell line was available that increased *FSHB* in response to activin. Therefore, the development of a differentiated gonadotrope cell line was critical for further understanding of what controls FSH production.

A. LβT2 Cells: Transformed Mouse Gonadotropes

In 1996, evidence for the existence of the first gonadotrope cell line was reported. This cell line was derived from a pituitary tumor induced by targeted oncogenesis in transgenic mice (119), a technique often used to immortalize specific cell types. Gonadotrope-specific targeting occurred through the use of the rat *LHB* promoter linked to the SV40 T-antigen oncogene, and only one of the cell lines generated, LβT2, seemed representative of gonadotrope function. It expressed *LHB*, as well as the α-subunit of LH and FSH (119). The expression of *FSHB*, however, was not observed. Not until three years later was it shown that in response to activin A, LβT2 cells synthesize *FSHB* and secrete FSH (120). Since that time, LβT2 cells have been shown to display many characteristics of

gonadotrope cells, including expression of receptors for GnRH and activin (ActRI and IB, and ActRII and IIB, [121]). They also express Smads and members of the MAPK pathway (39, 46, 63, 121, 122). Therefore, these were the first cells that made it possible to study the direct effects of activin and other hormones on *FSHB*.

LβT2 cells are also useful for studying transcriptional regulation of *FSHB* in other species. An o*FSHB* reporter that consists of 4.7 kb of the o*FSHB* promoter plus 759 bp 3' to the transcriptional start site and the coding sequence for luciferase (o*FSHB*luc) can be expressed in gonadotropes of transgenic mice (123). LβT2 cells also express o*FSHB*luc, and the reporter is strongly induced by activin and also induced about 2-fold by GnRH (122). In addition, both rat and pig *FSHB* reporters are expressed in these cells and respond similarly to activin treatment (47, 61, 62).

Interestingly, both basal and GnRH-induced expression of o*FSHB*luc are inhibited by follistatin treatment (122), suggesting that these cells produce activin endogenously. In fact, they have been shown to express the activin β_B-, but not β_A-subunit (122), suggesting that activin B may stimulate low levels of *FSHB* transcription in these cells. The development of LβT2 cells was critical for discovering many of the intracellular mechanisms that control FSH production, as discussed in the previous sections of this review, and will continue to be important since it is the only cell line representative of gonadotropes.

B. Primary Mouse Gonadotropes

Although LβT2 cells are widely used to study *FSHB* regulation, they are transformed cells that were derived during embryogenesis, so they are not necessarily normal gonadotropes nor are they fully mature. Therefore, LβT2 cells may not always reflect true

gonadotrope function. Our laboratory thus developed a novel method to purify gonadotropes from mouse pituitaries. To do this, our laboratory created a construct homologous to o*FSHB*luc, but substituted the H-2K^K gene for luciferase (o*FSHB* H-2K^K) (124). Like o*FSHB*luc, o*FSHB* H-2K^K is targeted to gonadotropes of transgenic mouse pituitaries, thus only gonadotropes express the cell surface antigen, H-2K^K. Using a double antibody method, followed by magnetic separation, these unique gonadotropes can be isolated, with the overall efficiency of isolation ranging from 50 - 95 %, from all other pituitary cell types to a purity level of 95 to 99 % (124, 125).

Unlike LβT2 cells, these primary gonadotropes synthesize *FSHB* and secrete FSH on their own, in the absence of activin (124). As expected, activin robustly increases FSH production even further, and follistatin inhibits it (124). Interestingly, culturing these cells with non-gonadotropes increased *FSHB* expression to a level higher than that observed with activin treatment. This suggested that paracrine factors from non-gonadotropes exert a high level of control on FSH. These factors, however, are currently unidentified. Development of this purification strategy was a critical milestone in gonadotrope-based research by providing a method to study *FSHB* regulation in primary gonadotropes for the first time, and importantly, provided a way to validate results in LβT2 cells by comparison with primary gonadotropes.

C. A Difference Between LβT2 and Primary Mouse Gonadotropes

Activin is the most potent inducer of *FSHB* expression, so it is likely that treatment with TGFβ would elicit similar stimulatory effects because both activin and TGFβ utilize the same intracellular signaling mechanisms, such as the Smad pathway (see section V.D).

However, this was not found to be the case. Through a collaborative effort, our laboratory found that in LβT2 cells, TGFβ, unlike activin, is incapable of stimulating a mouse *FSHB* reporter construct, nor can it promote phosphorylation of either Smad2 or 3 (125). This was due to the fact that LβT2 cells do not express TGFβ type II receptors (125), a finding that was later confirmed by other investigators (126). Using the purified, primary mouse gonadotropes, we found that they do express functional TGFβ type II receptors (125). Surprisingly, TGFβ inhibits *FSHB* expression by 95 %, while activin causes a 31-fold increase (125). Because both activin and TGFβ can activate Smad2 and 3, this novel finding provides further support for the involvement of an alternate and unidentified transcription factor. Clearly, Smads alone are not sufficient to regulate *FSHB* transcription. If so, then both activin and TGFβ would have elicited similar and not differential effects on *FSHB*. This study also showed that LβT2 cells are not entirely representative of normal and mature gonadotropes. The primary gonadotropes will thus be critical for not only future work on *FSHB* regulation, but also validation of findings in LβT2 cells.

IX. Summary of Relevant Dissertation Work

The following chapters (I-IV) presented in this dissertation have significantly advanced knowledge about the molecular mechanisms that govern activin-mediated transcription of *FSHB*. In Chapter I a major discovery was made, showing that activin and TGFβ have opposing actions on mouse *FSHB* expression in primary mouse gonadotropes (Chapter I, Fig. 7A). This finding was a first indication that *FSHB* transcription likely depended on a transcription factor other than just Smad2 and/or 3 because both are known to be activated by activin and TGFβ. Importantly, Chapter II showed that receptor-mediated

activation of Smad3 was not important for activin induction of ovine *FSHB* (o*FSHB*) and that the kinetics of o*FSHB* induction were atypical of a Smad-dependent response (Chapter II, Fig. 3). Although these findings correlated with our hypothesis that Smads were unlikely to be the only players in *FSHB* regulation by activin, the role of Smads 2 and 4 remained untested.

The work presented in Chapter III suggested that Smad 2 and/or 4 may be involved because a putative SBE from -162 bp to -159 bp was important for activin induction of an o*FSHB* promoter/reporter in LβT2 cells (Chapter III, Fig. 2). In addition, we identified a novel DNA sequence juxtaposed upstream to this SBE that was physiologically important for o*FSHB* expression (Chapter III, Fig. 3). The DNA sequence was discovered to be a putative binding site for Runx and FOX protein families since they bind similar sequences both can interact with Smads. Therefore, it is possible that either a Runx or FOX protein may bind to this newly identified site in a Smad 2 or 4-associated and activin-dependent manner. First, however, it was necessary to define the role of Smad2 and/or 4 and identify a candidate Smad binding partner important that may be for activin regulation of o*FSHB*.

The work presented in Chapter IV showed that Smad4 is capable of binding the -162 bp single-copy SBE (Chapter IV, Fig. 1B) and that Smad4, but not Smad2 is important for o*FSHB* induction (Fig. 2). Moreover, we found that activin induction of o*FSHBLuc* occurs biphasically, suggesting the possible involvement of a rapidly activated transcription factor gene that would be required for the second phase of o*FSHB* induction. We identified one candidate, *FOXQ1* that was logical for three reasons. First, because of its rapid and transient induction by activin (Fig. 4). Second, because of the importance of a putative FOX binding

site in the o*FSHB* promoter upstream to a SBE that binds Smad4 (Fig. 1B), and third, because Smad4 is not known to transactivate genes independent of other transcription factors. Collectively, this dissertation has shown the importance of Smad4, but not Smad2 or 3 in regulating activin induction of o*FSHB*, and that *FSHB* transcription likely depends on a regulated transcription factor, such as FOXQ1. As mentioned above, it is possible that FOXQ1 may bind to the site we identified in conjunction with Smad4 to regulate activin-dependent transcription of o*FSHB*, but this is a hypothesis that needs further attention.

REFERENCES

1. Kumar, T.R., Wang, Y., Lu, N., and M.M. Matzuk. 1997. Follicle-stimulating hormone is required for ovarian follicle maturation but not male fertility. Mol. Endocrinol. 13, 851-865.

2. Fiddes, J.C., and H.M. Goodman. 1981. The gene encoding the common alpha subunit of the four human glycoprotein genes. J. Appl. Genet. 1, 3-18.

3. Jameson, J.L., Becker, C.B., Lindell, C.M., and J.F. Habener. 1988. Human follicle-stimulating hormone b-subunit gene encodes multiple messenger ribonucleic acids. Mol. Endocrinol. 2, 806-815.

4. Fauser, B.C.J.M., and A.M. van Heusden. 1997. Manipulation of human ovarian function: physiological concepts and clinical consequences. Endocrine Reviews. 18, 71-106.

5. Palermo, R. 2007. Differential actions of FSH and LH during folliculogenesis. Reprod. Biomed. Online. 15, 326-337.

6. Kumar, T.R., Low, M.J., and M.M. Matzuk. 1998. Genetic rescue of follicle-stimulating hormone β-deficient mice. Endocrinology. 139, 3289-3295.

7. Kumar, T.R., Palapattu, G., Wang, P., Woodruff, T.K., Boime, I., Byrne, M.C., and M.M. Matzuk. 1999. Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. Mol. Endocrinol. 13, 851-865.

8. Su, P., Wu, J.C., Sommer, J.R., Gore, A.J., Petters, R.M., and W.L. Miller. 2005. Conditional induction of ovulation in mice. Biol. Reprod. 73, 681-687.

9. Marshall, J.C., Dalkin A.C., Haisenleder, D.J., Paul, S.J., Ortolano, G.A., and R.P. Kelch. 1991. Gonadotropin-releasing hormone pulses: regulators of gonadotropin synthesis and ovulatory cycles. Recent Prog. Horm. Res. 47, 155-187.

10. Dalkin, A.C., Haisenleder, D.J., Ortolano, G.A., Ellis, T.R., and J.C. Marshall. 1989. The frequency of gonadotropin-releasing-hormone stimulation differentially regulate gonadotropin subunit messenger ribonucleic acid expression. Endocrinology. 125, 917-924.

11. Lariviere, S., Garrel, G., Simon, V., Soh, J., Laverriere, J., Counis, R., and J. Cohen-Tannoudji. 2007. Gonadotropin-releasing hormone couples to 3', 5'-cyclic adenosine-5'-monophosphate pathway through novel protein kinase Cδ and -ε in LβT2 gonadotrope cells. Endocrinology. 148, 1099-1107.

12. Shafiee-Kermani, F., Han, S., and W.L. Miller. 2007. Chronic gonadotropin-releasing hormone inhibits activin induction of the ovine follicle-stimulating hormone β-subunit: involvement of 3', 5'-cyclic adenosine monophosphate response element binding protein and nitric oxide synthase type I. Endocrinology. 148, 3346-3355.

13. Kanasaki, H., Bedecarrats, G.Y., Kam, K., Xu, S., and U.B. Kaiser. 2005. Gonadotropin-releasing hormone pulse frequency-dependent activation of extracellular signal-regulated kinase pathways in perifused LβT2 cells. Endocrinology. 146, 5503-5513.

14. Coss, D., Hand, C.M., Yaphockun, K.K.J., Ely, H.A., and P.L. Mellon. 2007. p38 mitogen-activated protein kinase is critical for synergistic induction of the FSHβ gene by gonadotropin-releasing hormone and activin through augmentation of c-Fos induction and Smad phosphorylation. Mol. Endocrinol. 21, 3071-3086.

15. Haisenleder, D.J., Burger, L.L., Walsh, H.E., Stevens, J., Aylor, K.W., Shupnik, M.A., and J.C. Marshall. 2008. Pulsatile gonadotropin-releasing hormone stimulation of gonadotropin subunit transcription in rat pituitaries: evidence for the involvement of Jun N-terminal kinase but not p38. Endocrinology. 149, 139-145.

16. Mutiara, S., Kanasaki, H., Harada, T., Oride, A., and K. Miyazaki. 2008. The involvement of phosphatidylinositol 3-kinase in gonadotropin-releasing hormone-induced gonadotropin alpha- and FSH beta-subunit genes expression in clonal gonadotroph LbetaT2 cells. Mol. Cell. Endocrinol. 283, 1-11.

17. Strahl, B.D., Huang, H-J., Sebastian, J., Ghosh, B.R., and W.L. Miller. 1998. Transcriptional activation of the ovine follicle-stimulating hormone β-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. Endocrinology. 139, 4455-4465.

18. Strahl., B.D., Huang, H-J., Pedersen, N.R., Wu, J.C., and W.L. Miller. 1997. Two proximal activating protein-1-bindin sites are sufficient to stimulate transcription of the ovine follicle-stimulating hormone-beta gene. Endocrinology. 138, 2621-2631.

19. Karin, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. 270, 16483-16486.

20. Vasilyev, V.V., Pernasetti, F., Rosenberg, S.B., Barsoum, M.J., Austin, D.A., Webster, N.J.G., and P.L. Mellon. 2002. Transcriptional activation of the ovine follicle-stimulating hormone-β gene by gonadotropin-releasing hormone involves multiple signal transduction pathways. Endocrinology. 143, 1651-1659.

21. Culler, M.D., and A. Negro-Vilar. 1986. Evidence that pulsatile follicle-stimulating hormone secretion is independent of endogenous luteinizing hormone-releasing hormone. Endocrinology. 118, 609-612.

22. Mason, A.J., Pitts, S.L., Nikolics, K., Szonyi, E., Wilcox, J.N., Seeburg, P.H., and T.A. Stewart. 1986. The hypogonadal mouse: reproductive functions restored by gene therapy. Science. 234, 1372-1378.

23. Naor, Z., Jabbour, H.N., Naidich, M., Pawson, A.J., Morgan, K., Battersby, S., Millar, M.R., Brown, P., and R.P. Millar. Reciprocal cross talk between gonadotropin- releasing hormone (GnRH) and prostaglandin receptors regulates GnRH receptor expression and differential gonadotropin secretion. Mol. Endocrinol. 21, 524-537.

24. Pawson, A.J., and A.S. McNeilly. 2005. The pituitary effects of GnRH. Animal Reprod. Sci. 75-94.

25. Massague, J., Cheifetz, S., Laiho, M., Ralph, D., Weis, F., and A. Zentella. 1992. Transforming growth factor-beta. Cancer Surv. 12, 81-103.

26. Chin, D., Boyle, G.M., Parsons, P.G., and W.B. Coman. What is transforming growth factor-beta (TGF-beta)? Br. J. Plast. Surg. 57, 215-221.

27. Itoh, S., Itoh, F., Goumans, M-J., and P. ten Dijke. 2000. Signaling of transforming growth factor-β family members through Smad proteins. Eur. J. Biochem. 267, 6954-6967.

28. Matzuk, M.M., Kumar, T.R., Vassalli, A., Bickenbach, J.R., Roop, D.R., and R., Jaenisch. 1995. Functional analysis of activins during mammalin development. Nature. 374, 354-356.

29. Bottinger, E.P, Letterio, J.J., and A.B. Roberts. 1997. Biology of TGF-beta in knockout and transgenic mouse models. Kidney Int. 51, 1355-1360.

30. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo,W., Karr, D., and J. Spiess. 1986. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature. 321, 776-779.

31. Ling, N., Ying, S.Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and R. Guillemin. 1986. A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem. Biophys. Res. Comm. 138, 1129-1137.

32. Weiss, J., Crowley Jr., W.F., Halvorson, L.M., and J.L. Jameson. 1993. Perifusion of rat pituitary cells with gonadotropin-releasing hormone, activin, and inhibin reveals distinct effects on gonadotropin gene expression and secretion. Endocrinology. 132, 2307-2311.

33. Bernard, D.J. 2004. Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone β subunit in mouse gonadotrope cells. Mol. Endocrinol. 18, 606-623.

34. Weiss, J., Guendner, M.J., Halvorson, L.M., and J.L. Jameson. 1995. Transcriptional activation of the follicle-stimulating hormone beta-subunit gene by activin. Endocrinology. 136, 1885-1891.

35. Jones, K.L, de Kretser, D.M., Patella, S., and D.J. Phillips. 2004. Activin A and follistatin in systemic inflammation. Mol. Cell Endocrinol. 225, 119-125.

36. Reis, F.M., Luisi, S., Carneiro, M.M., Cobellis, L., Federico, M., Camargos, A.F., and F. Petraglia. 2004. Activin, inhibin and the human breast. Mol. Cell Endocrinol. 225, 77-82.

37. Yu, J., Shao, L.E., Lemas, V., Yu, A.L., Vaughan, J., Rivier, J., and W. Vale. 1987. Importance of FSH-releasing protein and inhibin in erythrodifferentiation. Nature. 330. 765-767.

38. Ethier, J.F., and J.K. Findlay. 2001. Roles of activin and its signal transduction mechanisms in reproductive tissues. Reproduction. 121, 667-675.

39. Bernard, D.J., Lee, K.B., and M.M. Santos. 2006. Activin B can signal through both ALK4 and ALK7 in gonadotrope cells. Reprod. Biol. Endocrinol. 4, 52.

40. Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fendley, B.M., Mason, A.J., Chin, W.W., Schwall, R.H., and W. Vale. 1991. Evidence for an autocrine role of activin B within rat anterior pituitary cultures. Endocrinology. 128, 1682-1684.

41. Thompson, T.B., Cook, R.W., Chapman, S.C., Jardetzky, T.S., and T.K. Woodruff. 2004. Beta A versus beta B: is it merely a matter of expression? Mol. Cell Endocrinol. 225, 9-17.

41. Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and J. Massague. 1994. Mechanism of activation of the TGF-β receptor. Nature. 370, 341-347.

43. Matzuk, M.M., Kumar, T.R., and A. Bradley. 1995. Different phenotypes for mice deficient in either activins or activin receptor type II. Nature. 374, 356-360.

44. Wuytens, G., Verschueren, K., de Winter, J.P., Gajendran, N., Beek, L., Devos, K., Bosman, F., de Waele, P., Andries, M., van den Eijnden-van Raaij, A.J.M., Smith, J.C., and D. Huylebroeck. 1999. Identification of two amino acids in activin A that are important for biological activity and binding to the activin type II receptors. J. Biol. Chem. 274, 9821-9827.

45. Attisano, L, Carcamo, J., Ventura, F., Weis, F.M.B, Massague, J., and J.L. Wrana. Identificaiton of human activin and TGFβ type I receptors that form heteromeric kinase complexes with type II receptors. Cell. 75, 671-680.

46. Safwat, N., Ninomiya-Tsuji, J., Gore, A.J., and W.L. Miller. 2005. Transforming growth factor β-activated kinase 1 is a key mediator of ovine follicle-stimulating hormone β-subunit expression. Endocrinology. 146, 4814-4824.

47. Lee, K.B., Khivansara, V., Santos, M.M., Lamba, P., Yuen, T, Sealfon, S.C., and D.J. Bernard. 2007. Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone beta subunit transcription. J. Mol. Endocrinol. 38, 315-330.

48. Tsuchida, K., Nakatani, M., Yamakawa, N., Hashimoto, O., Hasegawa, Y., and H. Sugino. 2004. Activin isoforms signal through type I receptor serine/threonine kinase ALK7. Mol. Cell Endocrinol. 220, 59-65.

49. Souchelnytskyi, S., Nakayama, T., Nakao, A., Moren, A., Heldin, C-H., Christian, J.L., and P. ten Dijke. 1998. Physical and functional interaction of murine and *Xenopus* Smad7 with bone morphogenetic protein receptors and transforming growth factor-β receptors. J. Biol. Chem. 273, 25364-25370.

50. Shi, Y., and J. Massague. 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell. 113, 685-700.

51. Hata, A., Lagna, G., Massague, J., and A. Hemmati-Brivanlou. 1998. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev. 12, 186-197.

52. Zhang, S., Fei, T., Zhang, L., Zhang, R., Chen, F., Ning, Y., Han, Y., Feng, X-H., Meng, A., and Y-G. Chen. 2007. Smad7 antagonizes TGF-β signaling in the nucleus by interfering with functional Smad-DNA complex formation. Mol. Cell Biol. 27, 4488-4499.

53. Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and S.E. Kern. 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol. Cell. 1, 611-617.

54. Shi, Y., Wang, Y.F., Jayaraman, L., Yang, H., Massague, J., and N.P. Pavletich. 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell. 4, 585-594.

55. Nagarajan, R.P., Zhang, J., Li, W., and Y. Chen. 1999. Regulation of Smad7 promoter by direct association with Smad3 and Smad4. J. Biol. Chem. 274, 33412-33418.

56. Ishida, W., Hamamoto, T., Kusanagi, K., Yagi, K., Kawabata, M., Takehara, K., Sampath, T.K., Kato, M., and K. Miyazono. 2000. Smad6 is a Smad1/5-induced Smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. J. Biol. Chem. 275, 6075-6079.

57. Feng, X.-H., and R. Derynck. 2005. Specificity and versatility in TGF-β signaling through Smads. Annu. Rev. Cell Dev. Biol. 21, 659-693.

58. Yagi, K., Goto, D., Hamamoto, T., Takenoshita, S., Kato, M., and K. Miyazono. 1999. Alternatively spliced variant of Smad2 lacking exon 3. Comparison with wild-type Smad2 and Smad3. J. Biol. Chem. 274, 703-709.

59. Lamba, P., Santos, M.M., Philips, D.P., and D.J. Bernard. 2006. Acute regulation of murine follicle-stimulating hormone β subunit transcription by activin A. J. Mol. Endocrinol. 36, 201-220.

60. Suszko, M.I., Lo, D.J., Suh, H., Camper, S.A., and T.K. Woodruff. 2003. Regulation of the rat follicle-stimulating hormone β-subunit promoter by activin. Mol. Endocrinol. 17, 318-332.

61. Gregory, S.J., Lacza, C.T., Detz, A.A., Xu, S., Petrillo, L.A., and U.B. Kaiser. 2005. Synergy between activin A and gonadotropin-releasing hormone in transcriptional activation of the rat follicle-stimulating hormone-β gene. Mol. Endocrinol. 19, 237-254.

62. Suszko, M.I., Balkin, D.M., Chen, Y., and T.K. Woodruff. 2005. Smad3 mediates activin-induced transcription of follicle-stimulating hormone β-subunit gene. Mol. Endocrinol. 19, 1849-1858.

63. Dupont, J., McNeilly, J., Vaiman, A., Canepa, S., Combarnous, Y., and C. Taragnat. 2003. Activin signaling pathways in ovine pituitary and LβT2 gonadotrope cells. Biol. Reprod. 68, 1877-1887.

64. Kumar, T.R., Schuff, K.G., Nusser, K.D., and M.J. Low. 2006. Gonadotroph-specific expression of the human follicle stimulating hormone beta gene in transgenic mice. Mol. Cell Endocrinol. 247, 103-115.

65. Bailey, J.S., Rave-Harel, N., McGillivray, S.M., Coss, D., and P.L. Mellon. 2004. Activin regulation of the follicle-stimulating hormone β-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol. Endocrinol. 18, 1158-1170.

66. Su, P., Shafiee-Kermani, F., Gore, A.J., Jia, J., Wu, J.C., and W.L. Miller. 2007. Expression and regulation of the β-subunit of ovine follicle-stimulating hormone relies heavily on a promoter sequence likely to bind Smad-associated proteins. Endocrinology. 148, 4500-4508.

67. Wang, G., Long, J., Matsuura, I., He, D., and F. Liu. 2005. The Smad3 linker region contains a transcriptional activation domain. Biochem. J. 386, 29-34.

68. de Caestecker, M.P., Yahata, T., Wang, D., Parks, W.T., Huang, S., Hill, C.S., Shioda, T., Roberts, A.B., and R.J. Lechleider. 2000. The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. J. Biol. Chem. 275, 2115-2122.

69. Massague, J., and D. Wotton. 2000. Transcriptional control by the TGF-β/Smad signaling system. EMBO J. 19, 1745-1754.

70. Mazhawidza, W., Winters, S.J., Kaiser, U.B., and S.S. Kakar. 2006. Identification of gene networks modulated by activin in LβT2 cells using DNA microarray analysis. Histol. Histopathol. 21, 167-178.

71. Denissova, N.G., Pouponno, C., Long, J., He, D., and F. Liu. 2000. Transforming growth factor β-inducible independent binding of SMAD to the Smad7 promoter. PNAS. 97, 6397-6402.

72. Moustakas, A., and C.-H. Heldin. 2005. Non-Smad TGF-β signals. J. Cell Sci. 118, 3573-3584.

73. Chen, X., Rubock, M.J., and M. Whitman. 1996. A transcriptional partner for MAD proteins in TGF-β signaling. Nature. 383, 691-696.

74. Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and M. Whitman. 1997. Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature. 389, 85-89.

75. Ross, S., and C.S. Hill. 2007. How the Smads regulate transcription. Int. J. Biochem. Cell Biol. 40, 383-408.

76. Hanai, J., Chen, L.F., Kanno, T., Ohtani-Fujita, N., Kim, W.Y., Guo, W.H., Imamura, T., Ishidou, Y., Fukuchi, M., Shi, M.-J., Stavnezer, J., Kawabata, M., Miyazono, K., and Y. Ito. 1999. Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Cα promoter. J. Biol. Chem. 274, 31577-31582.

77. Suszko, M.I., Antenos, M., Balkin, D.M., and T.K. Woodruff. 2007. Smad3 and Pitx2 cooperate in stimulation of FSHβ gene transcription. Mol. Cell Endocrinol. 281, 27-36.

78. Lamba, P., Khivansara, V., D'Alessio, A.C., Santos, M.M., D.J. Bernard. 2008. Paired-like homeodomain transcription factors 1 and 2 regulate follicle-stimulating hormone beta-subunit transcription through a conserved cis-element. Endocrinology. 149, 3095-3108.

79. Zhang, Y., Feng, X.H., and R. Derynck. 1998. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β induced transcription. Nature. 394, 909-913.

80. Kang, Y., Chen, C., and J. Massague. 2003. A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 expression in epithelial cells. Mol. Cell 11, 915-926.

81. Feng, X.H., Lin, X., and R. Derynck. 2000. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-β. EMBO J. 19, 5178-5193.

82. Nicol, R., and E. Stavnezer. 1998. Transcriptional repression by v-Ski and c-Ski mediated by a specific DNA binding site. J. Biol. Chem. 273, 3588-3597.

83. Suzuki, H., Yagi, K., Kondo, M., Kato, M., Miyazono, K., and K. Miyazawa. 2004. c-Ski inhibits the TGF-β signaling pathway through stabilization of inactive Smad complexes on Smad-binding elements. Oncogene. 23, 5068-5076.

84. Liu, X., Sun, Y., Weinberg, R.A., and H.F. Lodish. 2001. Ski/Sno and TGF-β signaling. Cytokine Growth Factor Rev. 12, 1-8.

85. Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H.F., and R.A. Weinberg. 1999. SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor β signaling. PNAS. 96, 12442-12447.

86. ten Dijke, P., and C.S. Hill. 2004. New insights into TGFβ-Smad signalling. Trends Biochem. Sci. 29, 265-273.

87. Jakubowiak, A., Pouponnot, C., Berguido, F., Frank, R., Mao, S., Massague, J., and S.D. Nimer. 2000. Inhibition of the transforming growth factor β1 signaling pathway by the AML1/ETO leukemia-associated fusion protein. J. Biol. Chem. 275, 40282-40287.

88. Ji, C., Eickelberg, O., McCarthy, T.L., and M. Centrella. 2001. Control and counter-control of TGF-β activity through FAST and Runx (CBFa) transcriptional elements in osteoblasts. Endocrinology. 142, 3873-3879.

89. Attisano, L., Silvestri, C., Izzi, L., and E. Labbe. 2001. The transcriptional role of Smads and FAST (FoxH1) in TGFβ and activin signaling. Mol. Cell Endocrinol. 180, 3-11.

90. Yue, J., and K.M. Mulder. 2000. Activation of the mitogen-activated protein kinase pathway by transforming growth factor-β. Methods Mol. Biol. 142, 125-131.

91. Massague, J. 2000. How cells read TGF-β signals. Nat. Rev. Mol. Cell Biol. 1, 169-178.

92. Murphy, L.O., MacKeigan, J.P., and J. Blenis. 2004. A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration. Mol. Cell Biol. 24, 144-153.

93. Engel, M.E., McDonnell, M.A., Law, B.K., and H.L. Moses. 1999. Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription. J. Biol. Chem. 274, 37413-37420.

94. Yu, L., Hebert, M.C., and Y.E. Zhang. 2002. TGF-β receptor-activated p38 MAP kinase mediates Smad-independent TGF-β responses. EMBO J. 21, 3749-3759.

95. David, L., Mallet, C., Vailhe, B., Lamouille, S., Feige, J.J., and S. Bailly. 2007. Activin receptor-like kinase 1 inhibits human microvascular endothelial cell migration: potential roles for JNK and ERK. J. Cell Physiol. 213, 484-489.

96. Cocolakis, E., Lemay, S., Ali, S., and J.-J. Lebrun. 2001. The p38 MAPK pathway is required for cell growth inhibition of human breast cancer cells in response to activin. J. Biol. Chem. 276, 18430-18436.

97. de Guise, C., Lacerte, A., Rafiei, S., Reynaud, R., Roy, M., Brue, T., and J.-J. Lebrun. 2006. Activin inhibits the human Pit-1 gene promoter through the p38 kinase pathway in a Smad-independent manner. Endocrinology. 147, 4351-4362.

98. Brown, J.D., DiChiara, M.R., Anderson, K.R., Gimbrone, M.A. Jr., and J.N. Topper. 1999. MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. J. Biol. Chem. 274, 8797-8805.

99. Kretzschmar, M., Doody, J., Timokhina, I., and J. Massague. 1999. A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. Genes Dev. 13, 804-816.

100. Funaba, M., Zimmerman, C.M., and L.S. Mathews. 2002. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. J. Biol. Chem. 44, 41361-41368.

101. Roelen, B.A.J., Cohen, O.S., Raychowdhury, M.K., Chadee, D.N., Zhang, Y., Kyriakis, J.M., Alessandrini, A.A., and H.Y. Lin. 2003. Phosphorylation of threonine 276 in Smad4 is involved in transforming growth factor-β-induced nuclear accumulation. Am. J. Physiol. Cell Physiol. 285, 823-830.

102. Ohshima, T., and K. Shimotohno. 2003. Transforming growth factor-β-mediated signaling via the p38 MAPK pathway activates smad-dependent transcription through SUMO-1 modification of Smad4. J. Biol. Chem. 51, 50833-50842.

103. Sano, Y., Harada, J., Tashiro, S., Gotoh-Mandeville, R., Maekawa, T., and S. Ishii. 1999. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-β signaling. J. Biol. Chem. 13, 8849-8957.

104. Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K., and E. Nishida. 1999. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-β-induced gene expression. J. Biol. Chem. 274, 27161-27167.

105. Uchida, K., Suzuki, H., Ohashi, T., Nitta, K., Yumura, W., and H. Nihei. 2001. Involvement of MAP kinase cascades in Smad7 transcriptional regulation. Biochem. Biophys. Res. Commun. 289, 376-381.

106. Lee, K.-S., Hong, S.-H., and S.-C. Bae. 2002. Both the Smad and MAPK pathways play a crucial role in Runx2 expression following induction by transforming growth factor-β and bone morphogenetic protein. Oncogene. 21, 7156-7163.

107. Inman, G.J., Nicolas, F.J., and C.S. Hill. 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-β receptor activity. Mol. Cell. 10, 283-294.

108. Ling, N., Ying, S.Y., Ueno, N., Esch, F., Denoroy, L., and R. Guillemin. 1985. Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. PNAS. 82, 7217-7221.

109. Meunier, H., Rivier, C., Evans, R.M., and W. Vale. 1988. Gonadal and extragonadal expression of inhibin alpha, beta A, and beta B subunits in various tissues predicts diverse functions. PNAS. 85, 247-251.

110. Bilezikjian, L.M., Corrigan, A.Z., Blount, A.L., and W.W. Vale. 1996. Pituitary follistatin and inhibin subunit messenger ribonucleic acid levels are differentially regulated by local and hormonal factors. Endocrinology. 137, 4277-4284.

111. Vale, W., Hsueh, A., Rivier, C., and J. Yu. 1990. The inhibin/activin family of growth factors. Peptide Growth Factors and Their Receptors, Handbook of Experimental Pharmacology. 211-248. Eds. M.A. Sporn and A.B. Roberts. Heidelberg: Springer-Verlag.

112. Mathews, L.S., and W.W. Vale. 1991. Expression and cloning of an activin receptor, a predicted transmembrane serine kinase. Cell. 65, 973-982.

113. Mathews, L.S., Vale, W.W., and C.R. Kintner. 1992. Cloning of a second type of activin receptor and functional characterization in Xenopus embryos. Science. 255, 1702-1705.

114. Lewis, K.A., Gray, P.C., Blount, A.L., MacConell, L.A., Wiater, E., Bilezikjian, L.M., and W. Vale. 2000. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature. 404, 411-414.

115. Bilezikjian, L.M., Blount, A.L., Leal, A.M.O., Donaldson, C., Fischer, W., and W. Vale. 2004. Autocrine/paracrine regulation of pituitary function by activin, inhbin and follistatin. Mol. Cell Endocrinol. 225, 29-36.

116. Lee, B.L., Unabia, G., and G. Childs. 1993. Expression of follistatin mRNA by somaotropes and mammotropes early in the rat estrous cycle. J. Histochem. Cytochem. 41, 955-960.

117. Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and S. Shimasaki. 1991. Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. Endocrinology. 129, 815-822.

118. Thompson, T.B., Lerch, T.F., Cook, R.W., Woodruff, T.K., and T.S. Jardetzky. 2005. The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. Developmental Cell. 9, 535-543.

119. Alarid, E.T., Windle, J.J., Whyte, D.B., and P.L. Mellon. 1996. Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development. 122, 3319-3329.

120. Graham, K.E., Nusser, K.D., and M.J. Low. 1999. LβT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to active A. J. Endocrinol. 162, R1-R5.

121. Pernasetti, F., Vasilyev, V.V., Rosenberg, S.B., Bailey, J.S., Huang, H.-J., Miller, W.L., and P.L. Mellon. 2001. Cell-specific transcriptional regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell model. Endocrinology. 142, 2284-2295.

122. Turgeon, J.L., Kimura, Y., Waring, D.W., and P.L. Mellon. 1996. Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. Mol. Endocrinol. 10, 439-450.

123. Huang, H.-J., Sebastian, J., Strahl, B.D., Wu, J.C., and W.L. Miller 2001. The promoter for the ovine follicle-stimulating hormone-beta gene (FSHbeta) confers FSHbeta-like expression on luciferase in transgenic mice: regulatory studies in vivo and in vitro. 142, 2260-2266.

124. Wu, J.C., Su, P., Safwat, N., Sebastian, J., and W.L. Miller. 2004. Rapid, efficient isolation of murine gonadotropes and their use in revealing control of follicle-stimulating hormone by paracrine pituitary factors. Endocrinology. 145, 5832-5839.

125. Gore, A.J., Philips, D.P., Miller, W.L., and D.J. Bernard. 2005. Differential regulation of follicle stimulating hormone by activin A and TGFB1 in murine gonadotropes. Reproductive Biology and Endocrinology. 3:73.

126. Suszko, M.I., and T.K. Woodruff. 2006. Cell-specificity of transforming growth factor-β response is dictated by receptor bioavailability. J. Mol. Endocrinol. 36, 591-600.

CHAPTER I

Differential regulation of follicle stimulating hormone by activin A and TGFβ1 in murine gonadotropes

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Differential regulation of follicle stimulating hormone by activin A and TGFB1 in murine gonadotropes

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Abstract

Background: Activins stimulate the synthesis of follicle stimulating hormone (FSH) in pituitary gonadotropes, at least in part, by inducing transcription of its beta subunit (*Fshb*). Evidence from several laboratories studying transformed murine LbetaT2 gonadotropes indicates that activins signal through Smad-dependent and/or Smad-independent pathways, similar to those used by transforming growth factor beta-1 (TGFB1) in other cell types. Therefore, given common intracellular signaling mechanisms of these two ligands, we examined whether TGFBs can also induce transcription of *Fshb* in LbetaT2 cells as well as in purified primary murine gonadotropes.

Methods: Murine *Fshb* promoter-reporter (-1990/+1 m*Fshb*-luc) activity was measured in LbetaT2 cells treated with activin A or TGFB1, and in cells transfected with either activin or TGFB receptors. The ability of the ligands to stimulate phosphorylation of Smads 2 and 3 in LbetaT2 cells was measured by western blot analysis, and expression of TGFB type I and II receptors was assessed by reverse transcriptase polymerase chain reaction in both LbetaT2 cells and primary gonadotropes purified from male mice of different ages. Finally, regulation of endogenous murine *Fshb* mRNA levels by activin A and TGFB1 in purified gonadotropes and whole pituitary cultures was measured using quantitative RT-PCR.

Results: Activin A dose-dependently stimulated -1990/+1 m*Fshb*-luc activity in LbetaT2 cells, but TGFB1 had no effect at doses up to 5 nM. Similarly, activin A, but not TGFB1, stimulated Smad 2 and 3 phosphorylation in these cells. Constitutively active forms of the activin (Acvr1b-T206D) and TGFB (TGFBR1-T204D) type I receptors strongly stimulated -1990/+1 m*Fshb*-luc activity, showing that mechanisms down stream of Tgfbr1 seem to be intact in LbetaT2 cells. RT-PCR analysis of LbetaT2 cells and whole adult murine pituitaries indicated that both expressed *Tgfbr1* mRNA, but that *Tgfbr2* was not detected in LbetaT2 cells. When cells were transfected with a human TGFBR2 expression construct, TGFB1 acquired the ability to significantly stimulate -1990/+1 m*Fshb*-luc activity. In contrast to LbetaT2 cells, primary murine gonadotropes from young mice (8–10 weeks) contained low, but detectable levels of *Tgfbr2* mRNA and these levels increased in older mice (1 yr). A second surprise was the finding that treatment of purified primary gonadotropes with TGFB1 decreased murine *Fshb* mRNA expression by 95% whereas activin A stimulated expression by 31-fold.

Conclusion: These data indicate that TGFB1-insensitivity in LbetaT2 cells results from a deficiency in *Tgfbr2* expression. In primary gonadotropes, however, expression of *Tgfbr2* does occur, and its presence permits TGFB1 to inhibit *Fshb* transcription, whereas activin A stimulates it. These divergent actions of activin A and TGFB1 were unexpected and show that the two ligands may act through distinct pathways to cause opposing biological effects in primary murine gonadotropes.

Background

Follicle-stimulating hormone (FSH) synthesis, secretion, and action are critical for reproductive function in mammals, particularly in females [1-3]. FSH production is regulated by a variety of neuroendocrine, intra-pituitary, and gonadal factors. Arguably, the most potent and selective stimulators of FSH synthesis are the activins, members of the transforming growth factor beta (TGFB) superfamily. Within the anterior pituitary, activins (activin B, in particular) act in paracrine/autocrine fashion to induce expression of the FSH beta (*Fshb*) subunit [4-9], the rate-limiting step in mature FSH production.

Several other factors that regulate FSH synthesis appear to have their actions via synergy with or perturbation of endogenous activin signaling. For example, activins synergistically stimulate rat and sheep *Fshb* transcription with gonadotropin releasing hormone (GNRH1) via cross-talk between activin and GNRH1 signaling pathways as well as through regulation of GNRH1 receptor expression [10-13]. Testicular androgens regulate *Fshb* transcription both directly and indirectly, although these effects vary across species [14]. In sheep, the direct actions of androgens on transcription appear to require intact activin signal transduction mechanisms [15]. Follistatins (FST) inhibit FSH production by binding activins and blocking the latter from interacting with their cell surface receptors [16,17]. Similarly, gonadal inhibins suppress FSH synthesis via antagonism of activins; in this case through competition with activins for binding to activin type II receptors [18-22]. Thus, many of the endocrine and paracrine factors known to affect FSH production do so through an interaction with or disruption of activin signaling. These and other data [23-25] indicate that the activins are critical for normal FSH regulation.

Both activins and TGFBs bind hetero-tetrameric receptor complexes consisting of ligand specific type I and type II receptor serine/threonine kinases [26,27]. Activins bind one of two type II receptors, ACVR2A or ACVR2B, which then recruit and phosphorylate the activin type IB receptor, ACVR1B or ALK4. In analogous fashion, TGFB1 binds TGFBR2, which recruits and phosphorylates TGFBR1 (also known as ALK5). Once activated, ACVR1B and TGFBR1 can phosphorylate Smad2 and Smad3 on C-terminal serine residues [28,29], and can also activate TGFB-activated kinase 1 (TAK1) [30,31]

In rodents, activins stimulate *Fshb* subunit gene transcription through both immediate-early and indirect (delayed or late) signaling pathways [30,32-34]. There is evidence to implicate Smads in *Fshb* gene transcription because they are rapidly phosphorylated and trans-located to the nucleus rapidly following activin A treatment [32,35-37]. In rats and mice, interference with Smad2 or Smad3 signaling impairs activin A-regulated *Fshb* transcription [13,32,33,35,37]. However, these proteins seem to play less important roles in activin A-induced *Fshb* transcription in sheep and humans [30,33], where Smad-independent mechanisms mediated by TGFB-activated kinase 1 (TAK1) appear to be critical for the former.

Like activins, the TGFB isoforms 1, 2, and 3 also phosphorylate and activate Smad2, Smad3 and TAK1 [28,29,31]. TGFB1 is produced within rat pituitary lactotropes [38,39]. Therefore, it is possible that TGFBs, acting in a paracrine manner, may also stimulate rodent *Fshb* transcription in gonadotropes via a similar Smad2/3- and/or TAK1-dependent mechanism. If this occurs, however, how could gonadotrope cells discriminate intracellularly between activin and TGFB-generated signals, specifically with respect to FSH regulation? This is an important question, in light of the fact that various physiological mechanisms that have evolved to spatially and temporally restrict activin's actions do not affect TGFB signaling [16,40]. For example, ovarian inhibin B production and action during metestrus and diestrus are critical for the suppression of activin-stimulated FSH production at these times of the rat estrous cycle [41,42]. Inhibin B may play a similar role during the follicular phase of the human menstrual cycle [43,44]. In addition, FST is dynamically regulated in the pituitary across the rat estrous cycle and its patterns of expression appear to be critical for the proper timing of the secondary FSH surge on the morning of proestrus [45,46]. Neither the inhibins nor FST suppress TGFB1 actions [19,47-49]. Therefore, antagonism of activins' stimulation of FSH by these proteins could theoretically be circumvented by unfettered TGFB1 stimulation of Smad2/3- and/or TAK1-dependent signaling mechanisms. We, therefore, examined TGFB1-regulated expression of murine *Fshb* subunit transcription to determine whether or not gonadotropes have evolved a mechanism to discriminate between the activin and TGFB ligands.

Methods

Reagents and constructs

Human recombinant (rh-) TGFB1, rh-activin A, recombinant mouse (rm)-follistatin 288 were purchased from R&D systems (Minneapolis, MN). Dulbecco's modified Eagle medium (DMEM), Lipofectamine/Plus, Lipofectamine 2000, gentamycin, and Trizol were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS). The anti-Smad3 affinity purified rabbit polyclonal antibody was purchased from Zymed (South San Francisco, CA). Anti-Smad2/3 and phospho-Smad2 affinity purified rabbit polyclonal antibodies were purchased from Upstate Biotech (Waltham, MA). The phospho-Smad3 rabbit polyclonal antibody was a generous gift of Dr. Michael Reiss (Robert Wood

Johnson Medical School). Protease inhibitor tablets (CompleteMini) were purchased from Roche (Indianapolis, IN). Deoxynucleotide triphosphates (dNTPs), MMLV reverse transcriptase, random primer hexamers, and Taq polymerase were from Promega (Madison, WI). The -1990/+1 m*Fshb*-luc reporter and constitutively active HA-rat ALK4 (Acvr1b) were described previously [32]. HA-human TGFBR1(T204D) was provided by Dr. Peter Scheiffele (Columbia University). The 3TP-luc reporter and HA-human TGFBR2 expression construct were gifts of Dr. Joan Massague (Memorial Sloan Kettering Cancer Center).

Primary gonadotropes

Pituitaries from mice containing the ovine *FSHB-H2K*^{K} transgene were used for gonadotrope purification. Young mice (8–10 weeks old; 10–18 mice) or older mice (1 year old; 12 mice) were killed and their pituitaries were dispersed and the gonadotropes were purified as reported [50]. Cells that did not attach to the magnetic column were labeled "gonadotrope-depleted," while cells eluted from the column after removal of the magnetic field were labeled "gonadotropes." Cell counts were obtained for all cell types using a hemocytometer. Equal numbers of cells were cultured in medium 199 (Gibco) with 10% charcoal-treated sheep serum and antibiotics/antimycotics as reported [51]. Gonadotropes and gonadotrope-depleted cells purified from younger mice were plated in triplicate at a density of 18,000 cells per well (first experiment) or 30,000 cells per well (second and third experiments). Cells isolated from older mice were plated in triplicate at a density of 50,000 cells per well in two separate experiments. For treatments with activin A or TGFB1, purified gonadotropes or whole pituitary cells were plated in triplicate at a density of 10,000 cells per well in three separate experiments. Gonadotropes, gonadotrope-depleted cells, and whole pituitary cells were cultured in 200 μl of media in 96 well Primaria culture plates (Becton Dickinson & Co, Franklin Lakes, NJ). Cells were incubated at 37° under 5% CO_2 for 48 hrs prior to RNA isolation. All mice were handled in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of North Carolina State University.

Cell culture and transfection

Immortalized murine gonadotrope LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego) and were cultured as described previously [32]. Murine fibroblast NIH3T3 cells were obtained from Dr. Patricia Morris (Population Council) and were cultured in DMEM/10% FBS. Cells were plated in 6- or 24-well plates at densities of 1 × 10^6 or 2 × 10^5 cells per well, respectively, approximately 36 hr prior to transfection. Cells were transfected with Lipofectamine/Plus or Lipofectamine 2000 following the manufacturer's instructions. Reporter plasmids were transfected at 1 μg (6-well) or 450 ng (24-well) per well. Expression plasmids were introduced at 300 ng (24-well) per well. In all experiments, the total amount of DNA added was balanced across treatments with empty expression vector pcDNA3.0 (Invitrogen).

In reporter experiments including ligand treatment, activin A or TGFB1 were added at the indicated concentrations for approximately 24 hr. Cells were washed with 1× PBS and lysed in 1× Passive Lysis Buffer (Promega). Luciferase assays were performed on a Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA) as described [32]. All transfection conditions were performed in triplicate and each experiment performed 2–3 times.

Western blotting

LβT2 and NIH3T3 cells were seeded at 7 or 4 × 10^5 cells per well, respectively, in 6-well plates. After 24–48 hr., cells were washed with serum-free DMEM and then incubated in the same medium overnight. The following day, cells were treated with the indicated concentrations of activin A or TGFB1 in fresh serum-free DMEM for 1 hr. After a wash with PBS, whole cell lysates prepared in RIPA buffer containing protease inhibitors. Equivalent amounts of protein were separated by 8% Tris-glycine SDS-PAGE and transferred to Protran (Schleicher & Schuell, Keene, NH). Filters were probed with anti-phospho-Smad2, anti-phospho-Smad3, anti-Smad2/3, or anti-Smad3 using previously described methods [32].

Semi-quantitative RT-PCR

Total RNA was extracted from adult female CD-1 murine pituitaries and LβT2 cells using Trizol following the manufacturer's instructions. Four μg of total RNA were reverse transcribed (RT) into cDNA using 100 ng random hexamer primers and 100 U MMLV-RT. A second set of samples was processed similarly, except the RT enzyme was omitted (no RT) as a control for contaminating genomic DNA in the RNA samples. One-tenth of each RT or RT-reaction was used as template in PCRs for *Tgfbr1* (503 bp) and *Tgfbr2* (536 bp). PCR was run using the following conditions for 35 cycles: 94C for 30 sec, 53C for 30 sec, and 72C for 30 sec. Reactions contained 0.4 pmol of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 2.5 U Taq polymerase. Following a final 7 min. extension step at 72C, one-fifth of each reaction was resolved on a 1 % agarose gel containing ethidium bromide. Gels were photo-documented using a digital camera interfaced with an IBM ThinkPad computer running the Kodak Digital Science 1D software (v.2.0.2) software. Reactions with no template (H_2O only) were used to confirm the absence of contaminating DNA in the reagents. The primer sets for *Tgfbr1* and *Tgfbr2* were as follows: *Tgfbr1*, (forward) AACCTGTTGTATTGCAGACTT and (reverse) GAGCAGAGT-

Table 1: Real Time RT-PCR primer and probe sequences

Primer/Probe Set		Sequence
Tgfbr1	Forward	5': CATTCACCACCGTGTGCCAAATGA
	Reverse	5': ACCTGATCCAGACCCTGATGTTGT
	Probe	5': AGATCGCCCTTTCATTTCAGAGGGCA
Tgfbr2	Forward	5': TCCCAAGTCGGATGTGGAAATGGA
	Reverse	5': TCGCTGGCCATGACATCACTGTTA
	Probe	5': AGCCCAGAAAGATGCATCCATCCACGTA
Prolactin	Forward	5': TCTCAAGGTCCTGAGGTGCCAAAT
	Reverse	5': CCATTGCACCCAAGCATGCACTGA
	Probe	5': ACAACTGCTAAACCCACATTCAGTCCA
Fshb	Forward	5': AGAGAAGGAAGAGTGCCGTTTCTG
	Reverse	5': ACATACTTTCTGGGTATTGGGCCG
	Probe	5': ATCAATACCACTTGGTGTGCGGGCTA
18s rRNA	Forward	5': GAAACTGCGAATGGCTCATTAA
	Reverse	5': GAATCACCACAGTTATCCAAGTAGGA
	Probe	5': ATGGTTCCTTTGGTCGCTCGCTCC

TCCCACGGTGT; *Tgfbr2*, (forward) TTGCCTGTGTGACTTCGGGCT and (reverse) CTATTTGGTAGTGTTCAGCGA.

Real-Time RT-PCR (RT-rtPCR)

Total RNA from primary and LβT2 cells was isolated and converted to cDNA as reported [51]. Oligonucleotides for Taqman real-time PCR were designed for murine cDNA using software from Integrated DNA Technologies, Inc (Coralville, IA) for *Tgfbr1*, *Tgfbr2*, *Fshb* and prolactin (Table 1). Using the same oligonucleotides as described previously [50], murine *18s* ribosomal RNA served as the endogenous control. All Taqman probes were 5' -labeled with FAM and real-time PCR of all cDNA samples was performed at the same time. Real-time PCR was performed in duplicate on triplicate cDNA samples from both gonadotropes and gonadotrope-depleted cells using an iCycler (Bio-Rad, Inc). Samples were incubated at 95°C for 3 min, and then for 40 complete cycles (95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec). There was a final extension step of 72°C for 3 min. Threshold cycle (C_T) values were determined with Bio-Rad software and used for relative quantitation with the $2^{-\Delta\Delta Ct}$ method [52].

Statistical analysis

The data from replicate luciferase assay experiments were highly similar and were pooled (n = 6 or 9 per treatment) for statistical analyses. Data are presented as fold-change from the control condition in each experiment. Differences between means were compared using one- or two-way analyses of variance followed by post-hoc Scheffe or Bonferroni tests (Systat 10.2, Richmond, CA). Comparisons of relative receptor mRNA expression in gonadotrope and gonadotrope-depleted cells in different age groups were performed with two-way ANOVAs of log-transformed data. *Fshb* mRNA levels were compared in one-way ANOVAs of log-transformed data. In all cases, significance was assessed relative to $p < 0.05$.

Results

Activin A but not TGFB1, stimulated Fshb *transcription in LβT2 cells*

LβT2 cells were transfected with a murine -1990/+1 *Fshb* luciferase promoter-reporter construct (-1990/+1 m*FSHB*-luc) [32] and were treated with different concentrations of activin A or TGFB1 for approximately 24 hr. Whereas activin A dose-dependently stimulated reporter activity, TGFB1 had no effect at concentrations up to 5 nM (Fig. 1A). In addition, activin A, but not TGFB1, stimulated Smad 2 and 3 phosphorylation in these cells (Fig. 1B). In contrast, the same lot of TGFB1 at lower concentrations (4–400 pM) dose-dependently stimulated the activin/TGFB responsive promoter of 3TP-luc [53], and Smad2/3 phosphorylation in murine NIH3T3 fibroblast cells (Figs. 2A and 2B). Thus, the TGFB1 ligand was biologically active, but LβT2 cells were somehow insensitive to it.

Constitutively active activin and TGFB type I receptors stimulated Fshb *transcription in LβT2 cells*

We previously showed that a constitutively active form of rat Acvr1b (T206D), which can stimulate Smad phosphorylation in the absence of activins and the type II receptors [54], stimulated murine *Fshb* promoter-reporter activity [32]. Here, we asked whether a constitutively active form of TGFBR1 (T204D; [55]) could similarly stimulate *Fshb* transcription in LβT2 cells. As shown in Figure 3, both rat Acvr1b-TD and human TGFBR1-TD potently stimulated -1990/+1m*Fshb*-luc. These data indicate that events downstream of TGFBR1 (whether Smad-dependent or Smad-independent; [30,56]) seem to be present in LβT2 cells

and therefore that the cells' insensitivity to TGFB1 likely derives from a deficiency at the receptor level.

LβT2 cells do not express the TGFB type II receptor, Tgfbr2

We used RT-PCR to examine *Tgfbr2* and *Tgfbr1* mRNA levels in LβT2 cells compared to adult murine pituitary glands. Whereas both LβT2 cells and pituitary glands expressed *Tgfbr1* mRNA, only the latter expressed *Tgfbr2* (Fig. 4).

Over-expression of TGFBR2 in LβT2 cells conferred TGFB1 responsiveness in LβT2 cells

RT-PCR analysis indicated that LβT2 cells do not express *Tgfbr2* mRNA. Therefore, these cells may not respond to TGFB1 because of a deficiency in this receptor. It is possible, however, that additional mechanisms contribute to TGFB1 insensitivity. To address this issue, we transfected LβT2 cells with a human TGFBR2 expression construct and examined TGFB1-stimulated *Fshb* transcription. Over-expression of the receptor alone had no effect on basal transcription, but made it possible for TGFB1 to stimulate -1990/+1 m*Fshb*-luc activity (Fig. 5). Therefore, a deficiency in *Tgfbr2* expression appeared to account for the inability of LβT2 cells to respond to TGFB1.

Primary murine gonadotropes exhibit low Tgfbr2 *expression*

LβT2 cells were derived from a pituitary tumor in a female transgenic mouse [57]. Whereas these cells show many of the features of fully differentiated gonadotropes, they are transformed cells and exhibit clear differences from gonadotropes *in vivo*. For example, basal *Fshb* expression is substantially lower in LβT2 cells than in gonadotropes (personal observations). Therefore, it is possible that the *Tgfbr2*-deficiency observed in LβT2 cells may not accurately reflect receptor expression in gonadotropes *in vivo*, though previous analyses in rats indicated that within the pituitary, *Tgfbr2* expression is most abundant in lactotropes [58,59]. In order to examine receptor expression in murine gonadotropes, we purified this cell type from male mice, aged eight to ten weeks (young) or 1 year of age (old), using a recently described transgenic model [50]. Using real-time RT-PCR, prolactin (*Prl*) expression was examined to determine the level of purification of the gonadotropes from mixed primary pituitary cultures as described earlier [50]. The level of purity ranged from 97 % to 99% (data not shown).

We then measured *Tgfbr1* and *Tgfbr2* mRNAs using real-time RT-PCR. *Tgfbr1* mRNA was significantly higher in older than younger animals ($p < 0.001$), but did not differ significantly between gonadotropes and gonadotrope-depleted cells, nor was there a significant interaction between these two variables (Fig. 6A). In contrast to LβT2 cell data, *Tgfbr2* mRNA was detected in gonadotropes but

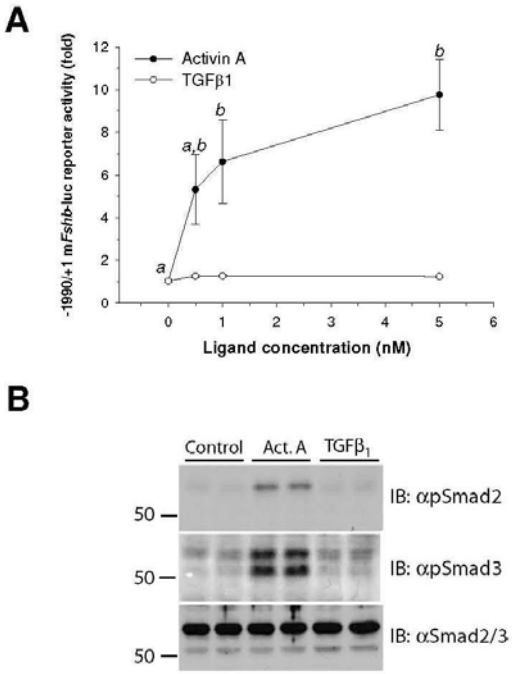

Figure 1
TGFB1 fails to stimulate *Fshb* transcription or Smad2/3 phosphorylation in LβT2 cells. A) LβT2 cells were seeded in 6-well plates and transfected with the murine -1990/+1 m*Fshb*-luc reporter. Following transfection, cells were treated with the indicated concentrations of activin A (closed circles) or TGFB1 for approximately 24 hours. Lysates were subjected to luciferase assays. Data points reflect mean (+/- SEM) fold-change in luciferase activity from the control condition (0 nM) in two experiments performed in triplicate (n = 6). Points with different letters differed significantly. B) LβT2 cells seeded in 6-well plates were treated with vehicle (control), 1.2 nM activin A or TGFB1 for 1 hour. Immunoblots (IB) of whole cell lysates were probed with rabbit anti-phospho-Smad2 (top), anti-phospho-Smad3 (middle), or anti-Smad2/3 (bottom) antibodies. Treatments were performed in duplicate. Numbers at the left are molecular weight standards in kDa.

it was greater in older than younger animals ($p < 0.001$). *Tgfbr2* mRNA was also higher in gonadotrope-depleted cells than pure gonadotropes across both age groups ($p < 0.007$) (Fig. 6B). In young mice, gonadotrope-depleted cells expressed *Tgfbr2* 6.5-fold higher than purified gonadotropes, whereas in the old mice the difference was reduced to 2.2-fold, but the interaction between cell type and age was not statistically significant. In the same assays, *Tgfbr2* was undetectable in LβT2 cells, and *Tgfbr1*

in LβT2 cells was expressed at roughly 20% of *Tgfbr1* in purified gonadotropes in young animals (data not shown).

Activin A increased, and TGFB1 decreased, Fshb *mRNA levels in murine gonadotropes and whole murine pituitary cultures*

Because purified gonadotropes express *Tgfbr2*, we used real-time RT-PCR to examine the effects of activin A and TGFB1 on endogenous *Fshb* mRNA levels. Treatment of purified gonadotropes from younger mice with activin A resulted in a 31-fold stimulation of *Fshb* mRNA (Fig. 7A). Surprisingly, treatment with TGFB1 resulted in a significant 95 % reduction in *Fshb* mRNA levels (Fig. 7A). Similar results were obtained in gonadotropes isolated from older mice (data not shown). Importantly, treatment with TGFB1 did not appear to affect cell viability since there were no differences in the levels of *18s* rRNA between control and treated cells (data not shown), and no morphological changes of treated cells relative to control were observed (personal observations). In whole pituitary cultures, activin A and TGFB1 exerted similar effects on *Fshb* expression to those seen in purified gonadotropes, although their magnitudes were reduced (Fig. 7B). Activin A induction was 19.5-fold, and inhibition by TGFB1 was only 56% and was not statistically significant ($p = 0.175$, Scheffe post-hoc). Therefore, unlike LβT2 cells, primary murine gonadotropes are sensitive to TGFB1 and the ligand inhibits *Fshb* mRNA levels, perhaps by repressing transcription.

Discussion

Activins regulate rodent and ovine *Fshb* transcription via Smad2/3- and/or TAK1-dependent intracellular signaling pathways [13,30,32,33,35,37,60]. Although TGFB isoforms also activate these pathways, we found that TGFB1 fails to regulate murine *Fshb* transcription in LβT2 cells, apparently because they do not express the TGFB type II receptor, Tgfbr2. However, when the cells were transiently transfected with the receptor, TGFB1 stimulated murine *Fshb* transcription. In striking contrast, we observed that gonadotrope cells purified from male mice expressed low levels of *Tgfbr2* mRNA and that TGFB1 suppressed *Fshb* mRNA in these cells as well as in mixed murine pituitary cell cultures. Because over-expression of TGFBR2 allowed TGFB1 to stimulate the -1990/+1 m*Fshb*-luc construct in LβT2 cells, differences in TGFB1 responses observed between the cell line and purified gonadotropes do not appear to be attributable to differences in *Tgfbr2* expression. Instead, the mechanisms through which TGFB1 inhibits *Fshb* expression in gonadotropes may be absent from LβT2 cells.

The identity of these inhibitory mechanisms is currently unknown, though opposing actions of activin A and

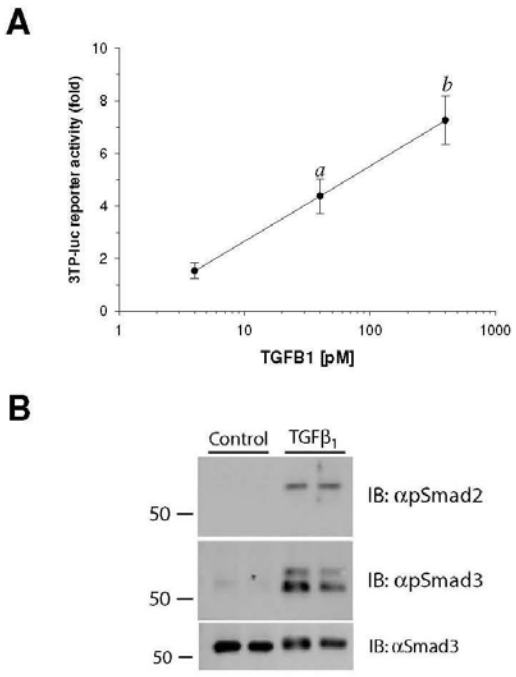

Figure 2
TGFB1 stimulates 3TP-luc activity and Smad2/3 phosphorylation in NIH3T3 cells. A) Murine fibroblast NIH3T3 cells were seeded in 24-well plates and transfected with the TGFB responsive promoter-reporter 3TP-luc. Following transfection, cells were treated with 4–400 pM TGFB1 for approximately 24 hours. Luciferase assays were performed as described. Data points reflect mean (+/- SEM) fold-change in luciferase activity from the control condition (0 pM, not pictured) in two experiments performed in triplicate (n = 6). The data are presented on a log-linear plot. Points with letters differed from control and points with different letters differed from one another. B) NIH3T3 cells seeded in 6-well plates were treated with vehicle (control) or 400 pM TGFB1 for 1 hour. Immunoblots on whole cell lysates were performed as described in the legend to Figure 1, except in the bottom blot an anti-Smad3 antibody was used in place of anti-Smad2/3. Treatments were performed in duplicate. Numbers at the left are molecular weight standards in kDa.

TGFB1 have been noted in other cellular contexts [61,62]. Also, TGFB1 can inhibit its own prototypic signaling via TGFBR1 and Smad2/3, through an ACVRL1 (ALK1)-dependent pathway [63]. That is, in addition to complexes containing two TGFBR2 and two TGFBR1 molecules, TGFB1 can form complexes with two TGFBR2, and

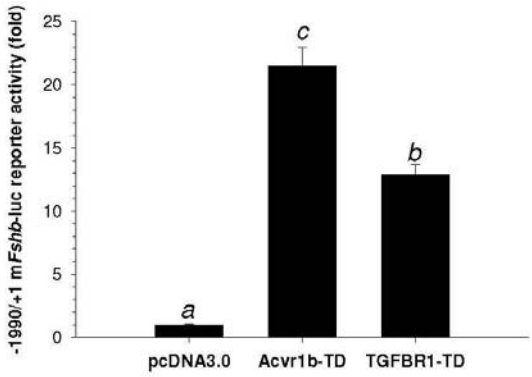

Figure 3
Constitutively active activin and TGFB type I receptors stimulate *Fshb* transcription in LβT2 cells. LβT2 cells seeded in 24-well plates were transfected with the -1990/+1 m*Fshb*-luc reporter and expression vectors for constitutively active forms of the rat activin (Acvr1b-TD) and human TGFB (TGFBR1-TD) type I receptors or with an empty expression vector (pcDNA3.0). Data reflect mean (+/- SEM) fold-change in luciferase activity from the control condition (pcDNA3.0) in two experiments performed in triplicate (n = 6). Bars with different letters differed significantly.

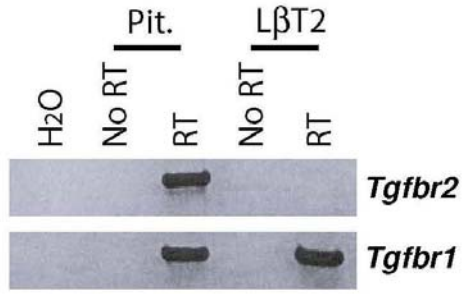

Figure 4
LβT2 cells do not express *Tgfbr2* mRNA. RT-PCR analysis of TGFB receptor expression in adult female CD-1 murine pituitary gland and LβT2 cells. Whereas *Tgfbr1* mRNA is expressed in both samples (bottom), *Tgfbr2* is expressed in whole pituitaries but not in LβT2 cells (top). No amplicons were detected in negative control samples (i.e., H_2O only or no RT).

one molecule each of TGFBR1 and ALK1. These latter receptor complexes can stimulate Smad1/5 phosphorylation and thereby inhibit TGFB1 actions mediated via TGFBR1 and Smad2/3 [63]. Whether or not LβT2 and/or gonadotropes express ALK1 has not been reported. However, it is possible that ALK1 expression in gonadotropes, but not LβT2 cells, may provide a mechanism for TGFB1 to antagonize endogenous activin B-dependent signaling (via Smad2/3) and hence decrease *Fshb* mRNA levels.

Although gonadotropes were purified to near homogeneity, it is possible that the inhibitory effects of TGFB1 were mediated indirectly through ligand action on contaminating cells in the cultures. For example, TGFB1 might stimulate FST synthesis by folliculostellate cells, which would then suppress the actions of endogenous activin B in gonadotropes [64]. However, if this were the mechanism of TGFB1 action, one might have anticipated greater inhibitory effects in the mixed rather than purified cultures where there are more folliculostellate cells, but the opposite was actually the case. That is, TGFB1 had greater suppressive activity in purified gonadotropes than in mixed cultures. Moreover, because activins stimulate FST production in primary pituitary cultures [65], one would predict that activin A would be less potent in stimulating

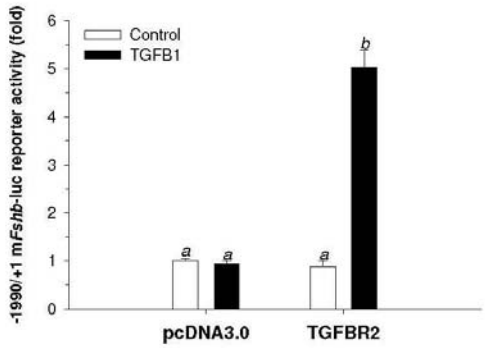

Figure 5
TGFBR2 over-expression rescues TGFB responsiveness in LβT2 cells. LβT2 cells seeded in 24-well plates were transfected with the -1990/+1 m*Fshb*-luc reporter and an expression vector for the human TGFBR2. Control wells were transfected with an empty expression vector, pcDNA3.0. Half of the cells in each condition were then treated with 400 pM TGFB1 for approximately 24 hr (filled bars). Data points reflect mean (+/- SEM) fold-change in luciferase activity from the control condition (pcDNA3.0, control) in three experiments performed in triplicate (n = 9). Bars with different letters differed significantly.

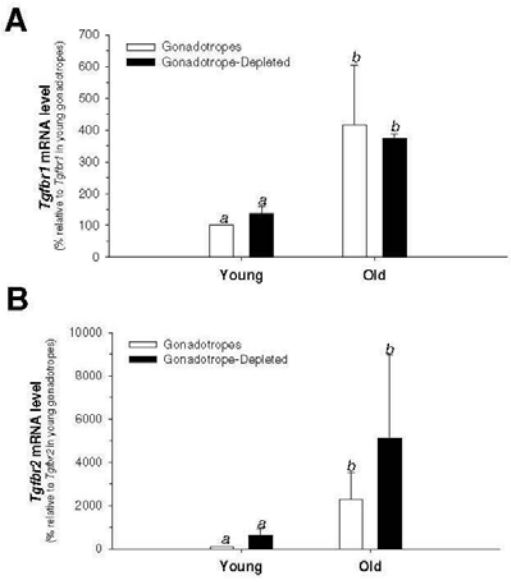

Figure 6

Purified murine gonadotropes express *Tgfbr2* at low levels. Purified gonadotropes and gonadotrope-depleted pituitary cells were plated in triplicate in 96 well culture plates. After 48 hrs, total RNA was isolated and real-time RT-PCR was performed to examine *Tgfbr1* and *Tgfbr2* mRNA expression. Normalized threshold cycle (Ct) values were averaged and used to compare receptor expression in the different cell types and different age groups using the $2^{-\Delta\Delta Ct}$ method for quantitation. Data are presented as mean (+ SD) (A) *Tgfbr1* or (B) *Tgfbr2* mRNA levels relative to those in young murine gonadotropes (set to 100%). Data from young and old animals were from 3 or 2 independent experiments, respectively. Bars with different letters differed significantly. When averaged across age-groups, *Tgfbr2* levels were higher in gonadotrope-depleted cells than in gonadotropes. Note the different scales of the *y*-axes in (A) and (B).

Fshb mRNA levels in mixed than in purified cultures and this was in fact what we observed (Fig. 7). Collectively, these data suggest that the effects of TGFB1 on *Fshb* mRNA levels are likely not mediated via regulation of FST production, though we cannot rule out that possibility entirely.

The finding that TGFB1 inhibited *Fshb* expression in primary murine gonadotropes and mixed pituitary cultures is novel, and appears to contrast with data reported previously for the rat and sheep *Fshb* genes. For example, TGFB was shown to potently and dose-dependently stimulate FSH secretion from rat primary pituitary culture [66]. However, the TGFB preparation used in that study, which was purified from human platelets [67], did not function similarly to recombinant TGFB1 in similar assays [39,68]. Subsequent reports have failed to show major effects of TGFB1 on FSH in any dispersed pituitary culture. For example, TGFB1 did not affect ovine *Fshb* promoter-reporter activity in transgenic mice [51] or FSH secretion from rat primary pituitary cultures [39]. In addition, only minor stimulation was observed in primary ovine pituitary cultures [69].

Finally, it is also notable that we observed age-dependent increases in *Tgfbr2* mRNA levels in purified gonadotropes. These data suggest that as mice mature, their gonadotropes may become more sensitive to the effects of TGFBs. The physiological significance, if any, of this change in receptor expression is not yet known, but is the subject of ongoing investigations. These data are nonetheless important in that they suggest that the low levels *Tgfbr2* mRNA detected in gonadotropes from young mice are likely not due to contamination by other cell types. Instead, it appears that as gonadotropes age, the level of *Tgfbr2* expression increases. Consistent with this notion is the lack of this receptor in LβT2 cells, which are thought to represent gonadotropes at an early stage of development. However, LβT2 cells were derived from a female mouse and the purified gonadotropes examined here were all from male mice, so it is possible that differences in *Tgfbr2* mRNA levels may also reflect sex differences in receptor expression.

Conclusion

The data reported here show that immortalized LβT2 cells lack the TGFB type II receptor, Tgfbr2, whereas the receptor appears to be expressed and functional in gonadotropes from male mice. As a result, TGFB1 (and likely all TGFB isoforms) is unable to regulate *Fshb* in LβT2 cells, but can inhibit transcription in primary murine gonadotropes. Because activins and TGFBs similarly activate Smads and TAK1, and both pathways contribute to activin A's stimulation of *Fshb* in rodents, it is surprising that TGFB would produce opposite effects to those of activins in purified gonadotropes. Nonetheless, as predicted, gonadotropes have evolved mechanisms for discriminating between the two classes of ligands. In the future, it will be critical to determine the mechanisms through which TGFBs inhibit *Fshb* in gonadotropes, particularly as animals age and *Tgfbr2* expression increases. Importantly, the data presented here suggest that LβT2 cells may not provide the best model system in which to pursue this aspect of FSH regulation.

Competing interests

The author(s) declare that they have no competing interests.

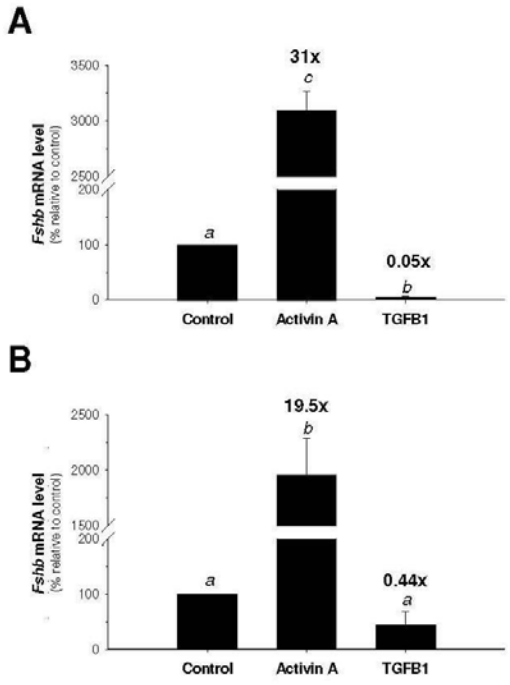

Figure 7

Activin A stimulates, and TGFB1 inhibits, *Fshb* transcription in gonadotropes. Purified gonadotropes (A) or whole pituitary cells (B) were plated in triplicate for control, activin A, or TGFB1 treatments in 96 well culture plates. After 24 hrs, cells were pre-treated with follistatin (250 ng/ml). After 24 hrs, all media was removed, and the cells were treated with control media, or media containing either activin A (60 ng/ml) or TGFB1 (60 ng/ml). After 24 hrs of treatment, total RNA was isolated and real-time RT-PCR was performed. Normalized threshold cycle (Ct) values were averaged and used to quantitate *Fshb* mRNA expression with the $2^{-(\Delta\Delta Ct)}$ method for quantitation. The mRNA levels were quantitated relative to the mRNA levels of *Fshb* in control cells for purified gonadotropes (A) or whole pituitary cells (B), which were normalized to 100 %. Plotted are the means (+/- SEM) for three experimental replicates. Bars with different letters differed significantly.

Authors' contributions

AJG participated in the design of the study, performed all of the gonadotrope purification and real-time RT-PCR analyses, and drafted portions of the manuscript. DPP conducted RT-PCR and receptor expression analyses. WLM participated in the design of the study and critically revised the manuscript. DJB participated in the design of the study, performed many of the transfection and western blot experiments and analyses, and drafted significant portions of the manuscript. All authors read and approved the final manuscript.

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References

1. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P: **Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance.** *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**:13612-13617.
2. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM: **The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction.** *Endocrinology* 2000, **141**:1795-1803.
3. Kumar TR, Wang Y, Lu N, Matzuk MM: **Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.** *Nature Genetics* 1997, **15**:201-204.
4. Corrigan AZ, Bilezikjian LM, Carroll RS, Bald LN, Schmelzer CH, Fendly BM, Mason AJ, Chin WW, Schwall RH, Vale W: **Evidence for an autocrine role of activin B within rat anterior pituitary cultures.** *Endocrinology* 1991, **128**:1682-1684.
5. Baratta M, West LA, Turzillo AM, Nett TM: **Activin Modulates Differential Effects of Estradiol on Synthesis and Secretion of Follicle-Stimulating Hormone in Ovine Pituitary Cells.** *Biol Reprod* 2001, **64**:714-719.
6. Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, Vale W, Sawchenko P: **Production and regulation of inhibin subunits in pituitary gonadotropes.** *Endocrinology* 1989, **124**:552-554.
7. Li MD, Macdonald GJ, Wise T, Ford JJ: **Positive Association between Expression of Follicle-Stimulating Hormone ß and Activin ßB-Subunit Genes in Boars.** *Biol Reprod* 1998, **59**:978-982.
8. Demura R, Suzuki T, Yajima R, Tajima S, Kubo O, Yoshimoto T, Demura H: **Inhibin alpha, beta A and beta B subunit messenger ribonucleic acid levels in cultured rat pituitary: studies by a quantitative RT-PCR.** *Endocrine Journal* 1996, **43**:403-410.
9. DePaolo LV, Bald LN, Fendly BM: **Passive immunoneutralization with a monoclonal antibody reveals a role for endogenous activin-B in mediating FSH hypersecretion during estrus and following ovariectomy of hypophysectomized, pituitary-grafted rats.** *Endocrinology* 1992, **130**:1741-1743.
10. Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL: **Cell-specific transcriptional regulation of follicle-stimulating hormone-beta by activin and gonadotropin-releasing hormone in the LbetaT2 pituitary gonadotrope cell model.** *Endocrinology* 2001, **142**:2284-2295.
11. Burger LL, Dalkin AC, Aylor KW, Haisenleder DJ, Marshall JC: **GnRH Pulse Frequency Modulation of Gonadotropin Subunit Gene Transcription in Normal Gonadotropes--Assessment by Primary Transcript Assay Provides Evidence for Roles of GnRH and Follistatin.** *Endocrinology* 2002, **143**:3243-3249.
12. Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J: **Gonadotropin-releasing hormone regulates follicle-stimulating hormone- beta gene expression through an**

activin/follistatin autocrine or paracrine loop. *Endocrinology* 1996, **137**:3667-3673.
13. Gregory SJ, Lacza CT, Detz AA, Xu S, Petrillo LA, Kaiser UB: **Synergy between Activin A and GnRH in Transcriptional Activation of the Rat Follicle-Stimulating Hormone beta (FSH{beta}) Gene.** *Mol Endocrinol* 2004:me.2003-473.
14. Burger LL, Haisenleder DJ, Dalkin AC, Marshall JC: **Regulation of gonadotropin subunit gene transcription.** *J Mol Endocrinol* 2004, **33**:559-584.
15. Spady TJ, Shayya R, Thackray VG, Ehrensberger L, Bailey JS, Mellon PL: **Androgen Regulates Follicle-Stimulating Hormone {beta} Gene Expression in an Activin-Dependent Manner in Immortalized Gonadotropes.** *Mol Endocrinol* 2004, **18**:925-940.
16. Harrison CA, Gray PC, Vale WW, Robertson DM: **Antagonists of activin signaling: mechanisms and potential biological applications.** *Trends Endocrinol Metab* 2005, **16**:73-78.
17. Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS: **The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding.** *Dev Cell* 2005, **9**:535-543.
18. Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM, Vale W: **Betaglycan binds inhibin and can mediate functional antagonism of activin signalling.** *Nature* 2000, **404**:411-414.
19. Martens JW, de Winter JP, Timmerman MA, McLuskey A, van Schaik RH, Themmen AP, de Jong FH: **Inhibin interferes with activin signaling at the level of the activin receptor complex in Chinese hamster ovary cells.** *Endocrinology* 1997, **138**:2928-2936.
20. Chapman SC, Bernard DJ, Jelen J, Woodruff TK: **Properties of inhibin binding to betaglycan, InhBP/p120 and the activin type II receptors.** *Mol Cell Endocrinol* 2002, **196**:79-93.
21. Xu J, McKeehan K, Matsuzaki K, McKeehan WL: **Inhibin antagonizes inhibition of liver cell growth by activin by a dominant-negative mechanism.** *J Biol Chem* 1995, **270**:6308-6313.
22. Cook RW, Thompson TB, Kurup SP, Jardetzky TS, Woodruff TK: **Structural Basis for a Functional Antagonist in the Transforming Growth Factor {beta} Superfamily.** *J Biol Chem* 2005, **280**:40177-40186.
23. Matzuk MM, Kumar TR, Bradley A: **Different phenotypes for mice deficient in either activins or activin receptor type II.** *Nature* 1995, **374**:356-360.
24. Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R: **Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction.** *Genes Dev* 1994, **8**:414-427.
25. Kumar TR, Agno J, Janovick JA, Conn PM, Matzuk MM: **Regulation of FSHbeta and GnRH receptor gene expression in activin receptor II knockout male mice.** *Mol Cell Endocrinol* 2003, **212**:19-27.
26. Thompson TB, Woodruff TK, Jardetzky TS: **Structures of an ActRIIB:activin A complex reveal a novel binding mode for TGF-beta ligand:receptor interactions.** *Embo J* 2003, **22**:1555-1566.
27. Hart PJ, Deep S, Taylor AB, Shu Z, Hinck CS, Hinck AP: **Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex.** *Nat Struct Biol* 2002, **9**:203-208.
28. Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL: **TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling.** *J Biol Chem* 1997, **272**:27678-27685.
29. Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P, Heldin CH: **Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling.** *J Biol Chem* 1997, **272**:28107-28115.
30. Safwat N, Ninomiya-Tsuji J, Gore AJ, Miller WL: **Transforming Growth Factor {beta}-Activated Kinase 1 Is a Key Mediator of Ovine Follicle-Stimulating Hormone {beta}-Subunit Expression.** *Endocrinology* 2005, **146**:4814-4824.
31. Javelaud D, Mauviel A: **Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis.** *Oncogene* 2005, **24**:5742-5750.
32. Bernard DJ: **Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells.** *Mol Endocrinol* 2004, **18**:606-623.
33. Lamba P, Santos MM, Philips DP, Bernard DJ: **Acute regulation of murine follicle-stimulating hormone beta subunit transcription by activin A.** *J Mol Endocrinol* 2005, **In press:**.
34. Weiss J, Guendner MJ, Halvorson LM, Jameson JL: **Transcriptional activation of the follicle-stimulating hormone beta-subunit gene by activin.** *Endocrinology* 1995, **136**:1885-1891.
35. Dupont J, McNeilly J, Vaiman A, Canepa S, Combarnous Y, Taragnat C: **Activin signaling pathways in ovine pituitary and LbetaT2 gonadotrope cells.** *Biol Reprod* 2003, **68**:1877-1887.
36. Norwitz ER, Xu S, Xu J, Spiryda LB, Park JS, Jeong KH, McGee EA, Kaiser UB: **Direct binding of AP-1 (Fos/Jun) proteins to a SMAD binding element facilitates both gonadotropin-releasing hormone (GnRH)- and activin-mediated transcriptional activation of the mouse GnRH receptor gene.** *J Biol Chem* 2002, **277**:37469-37478.
37. Suszko MI, Balkin DM, Chen Y, Woodruff TK: **Smad3 Mediates Activin-Induced Transcription of Follicle-Stimulating Hormone {beta}-Subunit Gene.** *Mol Endocrinol* 2005, **19**:1849-1858.
38. Burns G, Sarkar DK: **Transforming growth factor beta 1-like immunoreactivity in the pituitary gland of the rat: effect of estrogen.** *Endocrinology* 1993, **133**:1444-1449.
39. Sarkar DK, Kim KH, Minami S: **Transforming growth factor-beta 1 messenger RNA and protein expression in the pituitary gland: its action on prolactin secretion and lactotropic growth.** *Mol Endocrinol* 1992, **6**:1825-1833.
40. Phillips DJ: **Regulation of activin's access to the cell: why is mother nature such a control freak?** *Bioessays* 2000, **22**:689-696.
41. Woodruff TK, Krummen LA, Lyon RJ, Stocks DL, Mather JP: **Recombinant human inhibin A and recombinant human activin A regulate pituitary and ovarian function in the adult female rat.** *Endocrinology* 1993, **132**:2332-2341.
42. Woodruff TK, Besecke LM, Groome N, Draper LB, Schwartz NB, Weiss J: **Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner.** *Endocrinology* 1996, **137**:5463-5467.
43. Welt CK, Pagan YL, Smith PC, Rado KB, Hall JE: **Control of Follicle-Stimulating Hormone by Estradiol and the Inhibins: Critical Role of Estradiol at the Hypothalamus during the Luteal-Follicular Transition.** *J Clin Endocrinol Metab* 2003, **88**:1766-1771.
44. Welt CK, Hall JE, Adams JM, Taylor AE: **Relationship of Estradiol and Inhibin to the Follicle-Stimulating Hormone Variability in Hypergonadotropic Hypogonadism or Premature Ovarian Failure.** *J Clin Endocrinol Metab* 2005, **90**:826-830.
45. Halvorson LM, Weiss J, Bauer-Dantoin AC, Jameson JL: **Dynamic regulation of pituitary follistatin messenger ribonucleic acids during the rat estrous cycle.** *Endocrinology* 1994, **134**:1247-1253.
46. Besecke LM, Guendner MJ, Sluss PA, Polak AG, Woodruff TK, Jameson JL, Bauer-Dantoin AC, Weiss J: **Pituitary Follistatin Regulates Activin-Mediated Production of Follicle-Stimulating Hormone during the Rat Estrous Cycle.** *Endocrinology* 1997, **138**:2841-2848.
47. Chapman SC, Woodruff TK: **Modulation of activin signal transduction by inhibin B and inhibin-binding protein (InhBP).** *Mol Endocrinol* 2001, **15**:668-679.
48. Gonzalez-Manchon C, Vale W: **Activin-A, inhibin and transforming growth factor-beta modulate growth of two gonadal cell lines.** *Endocrinology* 1989, **125**:1666-1672.
49. Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H: **Activin-binding protein from rat ovary is follistatin.** *Science* 1990, **247**:836-838.
50. Wu JC, Su P, Safwat NW, Sebastian J, Miller WL: **Rapid, Efficient Isolation of Murine Gonadotropes and Their Use in Revealing Control of Follicle-Stimulating Hormone by Paracrine Pituitary Factors.** *Endocrinology* 2004, **145**:5832-5839.
51. Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL: **The promoter for the ovine follicle-stimulating hormone-beta gene (FSH-beta) confers FSHbeta-like expression on luciferase in transgenic mice: regulatory studies in vivo and in vitro.** *Endocrinology* 2001, **142**:2260-2266.

52. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDCt method.** *Methods* 2001, **25**:402-408.
53. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J: **TGF beta signals through a heteromeric protein kinase receptor complex.** *Cell* 1992, **71**:1003-1014.
54. Attisano L, Wrana JL, Montalvo E, Massague J: **Activation of signalling by the activin receptor complex.** *Mol Cell Biol* 1996, **16**:1066-1073.
55. Wieser R, Wrana JL, Massague J: **GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex.** *Embo J* 1995, **14**:2199-2208.
56. Yu L, Hebert MC, Zhang YE: **TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses.** *Embo J* 2002, **21**:3749-3759.
57. Alarid ET, Windle JJ, Whyte DB, Mellon PL: **Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice.** *Development* 1996, **122**:3319-3329.
58. Sarkar DK, Pastorcic M, De A, Engel M, Moses H, Ghasemzadeh MB: **Role of transforming growth factor (TGF)-beta Type I and TGF-beta type II receptors in the TGF-beta1-regulated gene expression in pituitary prolactin-secreting lactotropes.** *Endocrinology* 1998, **139**:3620-3628.
59. De A, Morgan TE, Speth RC, Boyadjieva N, Sarkar DK: **Pituitary lactotrope expresses transforming growth factor beta (TGF beta) type II receptor mRNA and protein and contains 125I-TGF beta I binding sites.** *J Endocrinol* 1996, **149**:19-27.
60. Suszko MI, Lo DJ, Suh H, Camper SA, Woodruff TK: **Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin.** *Mol Endocrinol* 2003, **17**:318-332.
61. Song Y, Keelan J, France JT: **Activin-A stimulates, while transforming growth factor beta 1 inhibits, chorionic gonadotrophin production and aromatase activity in cultured human placental trophoblasts.** *Placenta* 1996, **17**:603-610.
62. Hutchinson LA, Findlay JK, de Vos FL, Robertson DM: **Effects of bovine inhibin, transforming growth factor-beta and bovine Activin-A on granulosa cell differentiation.** *Biochem Biophys Res Commun* 1987, **146**:1405-1412.
63. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, ten Dijke P: **Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling.** *Mol Cell* 2003, **12**:817-828.
64. Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW: **Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin.** *Mol Cell Endocrinol* 2004, **225**:29-36.
65. Bilezikjian LM, Corrigan AZ, Vaughan JM, Vale WM: **Activin-A regulates follistatin secretion from cultured rat anterior pituitary cells.** *Endocrinology* 1993, **133**:2554-2560.
66. Ying SY, Becker A, Baird A, Ling N, Ueno N, Esch F, Guillemin R: **Type beta transforming growth factor (TGF-beta) is a potent stimulator of the basal secretion of follicle stimulating hormone (FSH) in a pituitary monolayer system.** *Biochem Biophys Res Commun* 1986, **135**:950-956.
67. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB: **Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization.** *J Biol Chem* 1983, **258**:7155-7160.
68. Murata T, Ying SY: **Transforming growth factor-beta and activin inhibit basal secretion of prolactin in a pituitary monolayer culture system.** *Proc Soc Exp Biol Med* 1991, **198**:599-605.
69. Chaidarun SS, Eggo MC, Stewart PM, Barber PC, Sheppard MC: **Role of growth factors and estrogen as modulators of growth, differentiation, and expression of gonadotropin subunit genes in primary cultured sheep pituitary cells.** *Endocrinology* 1994, **134**:935-944.

CHAPTER II

Transforming growth factor β-activated kinase 1 is a key mediator of ovine follicle-stimulating hormone β-subunit expression

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Transforming Growth Factor β-Activated Kinase 1 Is a Key Mediator of Ovine Follicle-Stimulating Hormone β-Subunit Expression

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FSH, a key regulator of gonadal function, contains a β-subunit (FSHβ) that is transcriptionally induced by activin, a member of the TGFβ-superfamily. This study used 4.7 kb of the ovine FSHβ-promoter linked to luciferase (oFSHβLuc) plus a well-characterized activin-responsive construct, p3TPLuc, to investigate the hypothesis that Smad3, TGFβ-activated kinase 1 (TAK1), or both cause activin-mediated induction of FSH. Overexpression of either Smad3 or TAK1 induced oFSHβLuc in gonadotrope-derived LβT2 cells as much as activin itself. Induction of p3TPLuc by activin is known to require Smad3 activation in many cell types, and this was true in LβT2 cells, where 10-fold induction by activin (2–8 h after activin treatment) was blocked more than 90% by two dominant negative (DN) inhibitors of Smad3 [DN-Smad3 (3SA) and DN-Smad3 (D407E)]. By contrast, 6.5-fold induction of oFSHβLuc by activin (10–24 h after activin treatment) was not blocked by either DN-Smad inhibitor, suggesting that activation of Smad3 did not trigger induction of oFSHβLuc. By contrast, inhibition of TAK1 by a DN-TAK1 construct led to a 50% decrease in activin-mediated induction of oFSHβLuc, and a specific inhibitor of TAK1 (5Z-7-Oxozeanol) blocked induction by 100%, indicating that TAK1 is necessary for activin induction of oFSHβLuc. Finally, inhibiting p38-MAPK (often activated by TAK1) blocked induction of oFSHβLuc by 60%. In conclusion, the data presented here indicate that activation of TAK1 (and probably p38-MAPK), but not Smad3, is necessary for triggering induction of oFSHβ by activin. (*Endocrinology* 146: 4814–4824, 2005)

FSH IS ESSENTIAL for female folliculogenesis and stimulates spermatogenesis in males (1). It is an α/β heterodimer made only in pituitary gonadotropes where transcription of its unique β-subunit (FSHβ) is paramount in determining overall FSH production. Transcription of FSHβ is controlled primarily by GnRH (2, 3) and gonadal- or pituitary-derived activins, inhibins, and follistatin (2–10).

Activin, a major inducer of FSHβ-transcription, seems central to FSH expression *in vivo*. It comprises three isoforms that are homo- or heterodimers of activin βA or βB chains (7). Activin A (homodimer of βA subunits) is the most potent inducer known for FSHβ (11), although other members of the TGFβ family have been shown to induce transcription of the FSHβ-subunit (5, 8). Recent studies suggest that activin action not only induces FSHβ but is also important for GnRH-mediated induction of FSHβ expression as well (12, 13).

Activin exerts its biological effects by binding to activin type II serine/threonine kinase receptors (ActRII or ActRIIB), which then bind and phosphorylate the activin type I receptor (ActRIB, also known as ALK4) (14–20). Once this occurs, the signal can be transmitted downstream by phosphorylating and activating Smad2 and/or Smad3. One or more of these Smads associate with a common signaling molecule, Smad4 (Co-Smad), to form a Smad2/4 or Smad3/4 complex that translocates to the nucleus, where it binds a specific DNA sequence known as a Smad binding element (SBE). The affinity of Smad complexes for DNA is low, but binding is usually stabilized by interaction(s) with adjacent tissue- and cell-specific transcription factors (21–28).

Individual or palindromic SBEs have been identified in both ovine and rat FSHβ-promoters, and they appear to be important for activin-mediated induction of FSHβ. Several are reported to bind Smad4. Also, the tale homeodomain of Pbx1 and Prep1 has been shown to partner with Smad4 within a DNA binding complex in the ovine FSHβ-promoter, and the same was shown for Pitx2c in the rat FSHβ-promoter (29, 30). Furthermore, overexpression of Smad3 (but not Smad2) has been shown to increase basal and activin-induced expression of FSHβ in LβT2 cells. Nevertheless, the binding of Smad4 to SBEs in the ovine and rat promoters of FSHβ is not significantly increased after treatment of cells with activin (29–31). Therefore, whereas Smad4 and its associated transcription factors appear to play pivotal roles in expression of FSHβ, no data actually prove that activin triggers FSHβ induction through the activation of Smad3 and subsequent binding to Smad4 and one or more FSHβ-SBEs.

Recently a Smad-independent pathway has been associated with the actions of several TGFβ family members [TGFβ, bone morphogenetic proteins (BMPs), activins] that use TGFβ-activated kinase 1 (TAK1), a member of the MAPK

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Abbreviations: BMP, Bone morphogenetic protein; Ca-ActRIB, constitutively active activin receptor IB; DN, dominant negative; αGSU, α-glycoprotein subunit; oFSHβLuc, ovine FSHβ-promoter linked to luciferase; JNK, c-Jun N-terminal kinase; MAPKKK, MAPK kinase kinase; SBE, Smad binding element; TAB, TAK1-binding protein; TAK1, TGFβ-activated kinase 1.

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kinase kinase (MAPKKK) family (32). Activation of TAK1 requires the association of TAK1-binding protein (TAB)1, which induces a conformational change in TAK1, allowing it to autophosphorylate its activation domain (33). The activated TAK1/TAB1 complex also binds TAB2 or TAB3 proteins, which modulate the regulatory effects of TAK1/TAB1 (34–38). Activation of TAK1 initiates MAP kinase cascades that have been shown to phosphorylate downstream c-Jun N-terminal kinase (JNK), ERK, and p38 (34, 39–46).

The studies presented here focused initially on the hypothesis that Smad3 induces expression of an ovine FSHβ-promoter-luciferase construct (oFSHβLuc) that contains all the regulatory sequences needed for normal cell-specific expression and regulation of FSHβ in mouse gonadotropes (47–50). The transformed gonadotrope cell line LβT2 was used as a model to study the signaling pathway mediating activin induction of FSHβ expression (51). Studies included comparisons with a well-characterized activin-responsive construct known to require Smad3 for induction, p3TPLuc (48). When it appeared that Smad3 was important for basal expression of oFSHβ but could not be proven to trigger activin-mediated induction of oFSHβLuc, the studies focused on the hypothesis that a novel Smad-independent pathway uses TAK1 activation to induce oFSHβLuc.

Materials and Methods

Reagents

Recombinant human activin A was obtained from R&D Systems, Inc. (Minneapolis, MN). MAPK inhibitors SB203580, SP600125, and PD98059 were purchased from Calbiochem (San Diego, CA). Recombinant human follistatin 288 was obtained from Dr. A. F. Parlow at the National Hormone and Pituitary Program (Torrance, CA), and 5Z-7-Oxozeanol (TAK1 inhibitor) was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Reporter plasmids and expression vectors

The ovine reporter plasmid oFSHβLuc has been previously described (52). Smad3 and dominant negative (DN) Smad3 [DN-Smad3 (D407E)] were obtained from Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan) (53). DN-Smad3 (3SA) was obtained from Dr. Theresa A. Guise (University of Texas, San Antonio, TX) (54). Expression constructs for TAK1 (55), TAB1 (55), TAB2 (36), TAB3 (34), and DN-TAK1-KN (56) were obtained from Dr. Jun Tsuji (North Carolina State University, Raleigh, NC). Constitutively active activin receptor IB (Ca-ActRIB) was obtained from Dr. J. Wrana (Mount Sinai Hospital, Ontario, Canada) (18). Mock plasmid (pCMV DNA), containing the CMV promoter with no luciferase reporter gene, was obtained from Promega Corp. (Madison, WI). Control plasmid (pGL3-control) containing the GL3 luciferase reporter driven by an SV40 promoter was purchased from Promega.

Culture and transient transfection of LβT2 gonadotropes

LβT2 cells (51) were grown at 37 C in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin G, and 100 μg/ml streptomycin under 95% air-5% CO_2. Cells were grown in 150-cm^2 flasks until they were confluent and then transferred to 96-multiwell plates (Falcon; Fisher Scientific, Raleigh, NC) at a concentration of 25,000 cells/well. Cells were cotransfected, 24 h later, in triplicate with 50 ng oFSHβLuc, p3TPLuc, pGL3-control and 50–150 ng pCMV using Fugene6 (Roche Molecular Biochemicals, Basel, Switzerland). After transfection (24 h), cells were treated with fresh media, with or without activin (100 ng/ml), for an additional 24 h. Cells were then lysed in 50 μl passive lysis buffer, and 20 μl was assayed for luciferase activity.

Real-time RT-PCR

Total RNA from LβT2 cells was isolated and converted to cDNA as reported (5). Oligonucleotides for taqman real-time PCR were designed for mouse FSHβ, LHβ, and the α-glycoprotein subunit (αGSU) common to both LH and FSH, using software from Integrated DNA Technologies, Inc. (Coralville, IA). Mouse 18s ribosomal RNA served as the internal control for quantifying all molecules as reported earlier (57). The taqman probe for FSHβ was reported previously (57), and the primers and probes for LHβ and αGSU were: LHβ forward, 5′-AATCCCGCTCCACACAGTACATGA-3′; LHβ reverse, 5′-TCAGCTCAGGAGGTGTCCATTGTT-3′; LHβ probe, 5′-TGCCTTCCTCTTCAATGAGCTCAAAGGCGA-3′; αGSU forward, 5′-AGATCGACAATCACCTGCCCAGAA-3′; αGSU reverse, 5′ AGGAACATGGACAGCATGACCAGA-3′; αGSU probe, 5′-TCCAGAGCTTGCAGAAGAGCTATGGA-3′.

Real-time PCR was performed in duplicate on triplicate cDNA samples from LβT2 cells using an i-Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Samples were incubated at 95 C for 3 min, and then for 40 complete cycles (95 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec). There was a final extension step of 72 C for 3 min. Threshold cycle values were determined with Bio-Rad software and used for relative quantization with the $2^{-\Delta\Delta Ct}$ method.

Luciferase assay

Cells were harvested by adding 1× passive lysis buffer (Promega, Madison, WI) as described above for LβT2 cells. Luciferase activity was measured in 40% of the cell lysate (20 μl) by adding 100 μl luciferase assay system from Promega. Luciferase activity was measured for 20 sec using a Monolight 2010 single tube luminometer (Analytical Luminescence Laboratory, San Diego, CA) or automated Victor-Light microplate luminometer (PerkinElmer, Boston, MA).

Antibodies

The rabbit antimouse TAK1 (554–579) and rabbit antimouse TAB1 (480–500) polyclonal antibodies (Upstate Biotechnology, Inc., Lake Placid, NY) were also used to detect endogenous TAK1 and TAB1 in LβT2 cells. Secondary antibody used for detection was antirabbit IgG (Calbiochem).

Western blotting

Analysis of TAK1 and TAB1 expression by Western blot was performed as previously described (55). Briefly, LβT2 cells were cultured in 6-well tissue culture plates with 1 million cells/well for 24 h. Cells were pretreated with follistatin-288 (250 ng/ml) for 16 h to minimize FSHβ expression (unpublished results by Dr. H. J. Huang, this laboratory), presumably by eliminating any effects of endogenously produced activin or activin-like activist (5). Cells were then washed and treated with or without activin A (100 ng/ml) for the indicated time points. Cells were washed once with PBS, pH 7.4 (PBS; 4 C) and collected in 1 ml PBS. Cells were centrifuged at 1000 × *g* for 5 min (4 C) and lysed in 50 μl of 0.5% Triton X-100 lysis buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 12.5 mM β-glycerophosphate; 1.5 mM $MgCl_2$; 2 mM EGTA; 10 mM NaF; 2 mM dithiothreitol; 1 mM sodium orthovanadate; 1 mM phenylmethylsulfonylfluoride; and 20 mM aprotinin). Cells were sonicated for 5 sec and centrifuged at 10,000 × *g* for 5 min, and the cleared lysates were fractionated on a 7% SDS-polyacrylamide gel. Proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated with antibodies, and antibody localization was visualized with horseradish peroxidase-conjugated antibodies to rabbit IgG using the enhanced chemiluminescence Western blotting system (ECL; Amersham).

Statistics

Data (see Fig. 7; Western blot) were obtained two times, and the clearest blot is shown. Data from all other experiments were replicated at least three times, and all samples were assayed in triplicate. Means ± SEM values are shown in all figures; data in all (with one exception; see Fig. 7) were analyzed using one-way ANOVA with Tukey's multiple comparison test according to the Prism version 4 (GraphPad Software, Inc., San Diego, CA).

Results

Transfected Smad3 increased basal and activin-induced expression of oFSHβLuc equally

To investigate the role of Smad3 in mediating oFSHβ induction by activin, LβT2 cells were cotransfected with oFSHβLuc and increasing amounts of Smad3 DNA (25, 50, 75, 100, and 125 ng) (Fig. 1). Cotransfection with Smad3 increased basal expression of oFSHβLuc by 89, 116, 206, 240, and 312%, respectively. Activin treatment of cultures with transfected Smad3 increased oFSHβLuc induction above control cultures. However, increased induction paralleled increased basal expression at all concentrations of transfected Smad3, with an average increase of 4.5 ± 0.3. The ratios of induced/basal expression at each level of Smad3 were: 4.6 ± 2.1, 5.6 ± 1.5, 4.2 ± 1.1, 4.6 ± 1.2, and 3.7 ± 1.0, respectively, for the 25- to 125-ng treatments. None of these were significantly different from each other, nor were they different from the 5.7 ± 1.6 ratio observed in control cultures not transfected with Smad3 (Fig. 1).

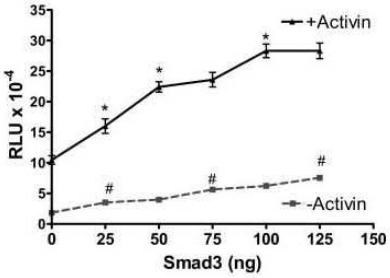


FIG. 1. Overexpression of Smad3 equally stimulated basal and activin-induced expression of oFSHβLuc. LβT2 cells were plated at 25,000 cells/well in 96-well tissue culture plates. After 24 h, they were cotransfected with 50 ng oFSHβLuc and increasing amounts of Smad3 expression construct (25, 50, 75, 100, and 125 ng). DNA amounts were kept constant at 200 ng DNA transfected per well using pCMV (mock plasmid) to balance amounts of DNA. After transfection (24 h), cells were treated with or without activin (100 ng/ml) for an additional 24 h and assayed for luciferase activity. Ratios of induced/basal were not significantly different from the 5.7 ± 1.6 ratio observed in control cultures not transfected with Smad3 ($P > 0.05$). One-way ANOVA/Tukey's was used to show that increasing levels of Smad3 increased luciferase expression; a significant increase between points ($P < 0.05$) was designated: #, for basal expression; *, for activin-stimulated expression. RLU, Relative light units.

Smad3 activation is required for induction of p3TPLuc but not for oFSHβLuc

To determine the functional significance of endogenous Smad3 signaling, two DN inhibitors of Smad3 [Smad3 (3SA) and Smad3 (D407E)] were tested. First, LβT2 cells were cotransfected with p3TPLuc, which can be induced by activin through a Smad3 pathway. Then p3TPLuc was cotransfected with one of two DN-Smad3 expression vectors to block the actions of endogenous Smad3 (see Fig. 2) (49, 53). Basal expression of p3TPLuc was not inhibited by either DN-Smad, but both inhibitors blocked 7-fold induction by activin by 87–96%. These data showed that DN-Smad3 (3SA) and DN-Smad3 (D407E) were effective inhibitors of activin-mediated activation of Smad3 in LβT2 cells.

Data (see Fig. 2B) show that DN-Smad3 (3SA) or DN-Smad3 (D407E) did not block basal or activin-mediated induction of oFSHβLuc. Activin induced oFSHβLuc expression by 5.1-fold, but neither Smad3 inhibitor altered the action of activin.

Subsequent results (see Fig. 2C) are similar, except that Ca-ActRIB was used to induce oFSHβLuc in LβT2 cells. The results show that cotransfection of Ca-ActRIB along with oFSHβLuc increased expression of oFSHβLuc by 9.5-fold. The DN inhibitors of Smad3, however, had no effect on this induction, just as observed for induction of oFSHβLuc with activin. These data show that the constitutively active activin receptor induced oFSHβLuc but that activation of Smad3 was not necessary for induction to occur.

Data indicate (see Fig. 2D) that there was no tendency for DN-Smad3 (3SA) to inhibit either basal or activin-mediated induction of oFSHβLuc over a range of concentrations (25–150 ng) higher and lower than the 50-ng amount used in Fig. 2, A–C. Activin increased expression of oFSHβLuc by 5.8-fold over basal expression, with or without DN-Smad3 (Fig. 2D).

Activin induction of oFSHβLuc and p3TPLuc have very different kinetics

The data in Fig. 3 show time-course data (24 h) for activin induction of oFSHβLuc and p3TPLuc. In Fig. 3A, activin A (100 ng/ml) had no observable effect on oFSHβLuc expression for 6 h. It then had a small, inductive effect from 8–12 h (~100% increase) before it increased expression linearly up to full induction at 22 h (520% increase). When DN-Smad3 (3SA) was cotransfected, the same pattern was observed, and there was no inhibition by DN-Smad3 (3SA) at any time during the 24-h period.

The data in Fig. 3B show that p3TPLuc was induced 6-fold at 2 h and 9-fold at 6 h and dropped to 2-fold by 10 h. Therefore, induction by activin was initiated well before 2 h and down-regulated shortly after 8 h. When cultures were cotransfected with DN-Smad3 (3SA), most of the induction between 2–10 h was blocked. Results shown with the DN-Smad3 in this time-course format indicated that activin induced expression of oFSHβ through a pathway that did not depend on Smad3 activation.

Altering expression of oFSHβLuc with TAK1, TAB1, TAB2, TAB3, and DN-TAK1

Next, the role of TAK1 as a potential mediator of activin induction was tested. LβT2 cells were transfected with either oFSHβLuc alone or with oFSHβLuc plus TAK1 and its binding proteins, TAB1, TAB2, or TAB3. Preliminary studies showed that overexpression of TAK1/TAB1 induced oFSHβLuc 45-fold, but this appeared to be nonspecific induction because it also increased expression of the SV40-driven pGL3Luc control construct and the minimal thymidine kinase luciferase construct, T109Luc, that are not activin responsive (data not shown). However, cotransfection of TAK1 with either TAB2 or TAB3 did increase expression of oFSHβLuc, just like activin (6.3-fold), and activin was unable to augment this induction much above 6.3-fold when added along with TAB2 or TAB3. Thus, transfections with TAK1 and either TAB2 or TAB3 nearly substituted for activin induction of

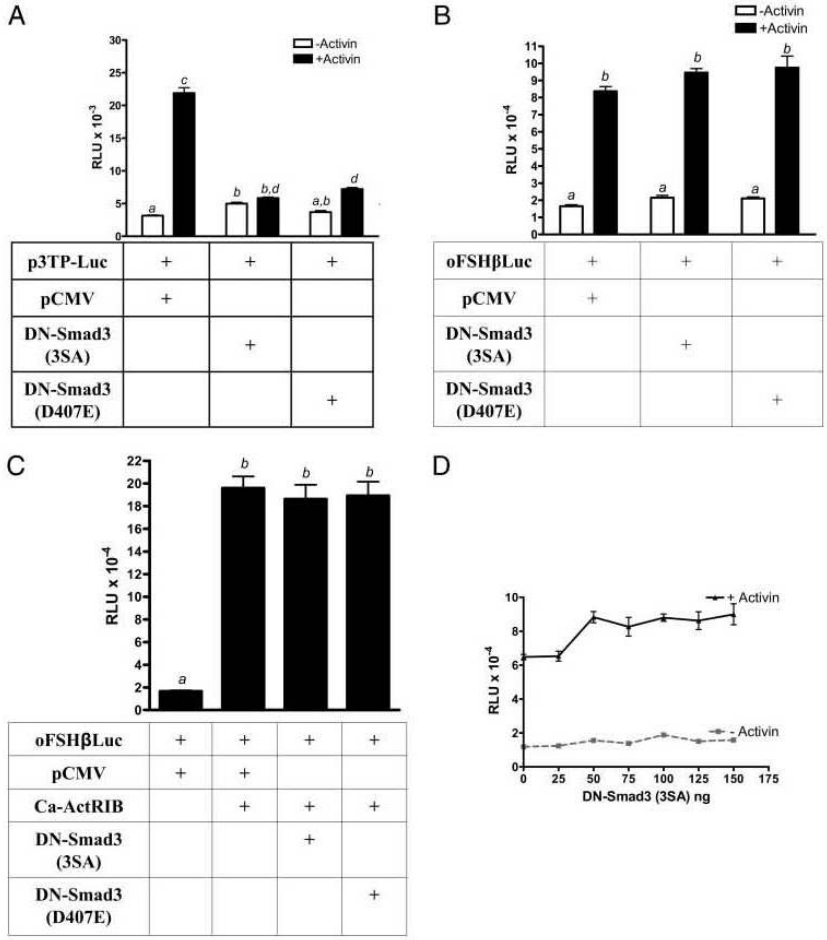


FIG. 2. Activin did not require activated Smad3 to induce oFSHβLuc expression. LβT2 cells were prepared and plated as in Fig. 1 and then treated as follows: A, Cells were cotransfected with 50 ng p3TPLux plus 50 ng pCMV, DN-Smad3 (3SA), or DN-Smad3 (D407E). After transfection (24 h), cells were treated with or without activin (100 ng/ml) for an additional 6 h and assayed for luciferase activity. *Means with different letters* are significantly different ($P < 0.05$) ($P < 0.001$ for c *vs.* d). B, Cells were treated as in A except that oFSHβLuc was used instead of p3TPLux. *Means with different letters* are significantly different ($P < 0.05$). C, Cells were transfected with 50 ng oFSHβLuc plus 100 ng pCMV (column 1) or 50 ng pCMV and 50 ng Ca-ActRIB (column 2). Columns 3 and 4 were transfected with 50 ng of either DN-Smad3 (SA) or DN-Smad3 (D407E) in place of pCMV. Cells were collected 24 h after transfection and assayed for luciferase activity. *Means with different letters* are significantly different ($P < 0.05$). D, Cells were cotransfected with 50 ng oFSHβLuc and increasing amounts of DN-Smad3 (3SA) expression construct (25, 50, 75, 100, 125, and 150 ng). Total DNA amounts were kept constant using mock plasmid pCMV. Twenty-four hours after transfection, cells were treated with or without activin (100 ng/ml) for an additional 24 h. Ratios of induced/basal were not significantly different from the 5.5 ± 0.22 ratio observed in control cultures not transfected with DN-Smad3 (3SA) ($P > 0.05$).

oFSHβLuc (Fig. 4A), suggesting they could be in the signaling pathway used by activin.

Transfections with TAK1/TAB2 or TAK1/TAB3 were tested with the pGL3Luc control expression vector, and the data in Fig. 4B show that its expression was not altered. Thus, overexpression of TAK1 in the presence of TAB2 or TAB3 was specific for inducing activin-responsive genes only.

To determine whether endogenous TAK1 was important for activin action, LβT2 cells were cotransfected with oFSHβLuc and a DN TAK1 mutant (DN-TAK1), which lacks the active site required for its kinase activity (32). This DN molecule (DN-TAK1) did suppress activin induction of oFSHβLuc by 50% (Fig. 4C). This finding was consistent with the concept that endogenous TAK1 is, at least partly, responsible for activin-mediated induction of oFSHβ.

Inhibiting activin induction of oFSHβLuc with a TAK1 inhibitor (5Z-7-Oxozeanol)

To further characterize the role of TAK1 in activin-mediated induction of oFSHβLuc, LβT2 cells were transfected with oFSHβLuc and then treated with increasing concentrations of 5Z-7-Oxozeanol, a newly discovered inhibitor of TAK1 that works by blocking the ATP binding site required for TAK1 kinase activity (55). Cells were treated with activin (100 ng/ml) and with or without 5Z-7-Oxozeanol for 24 h. Activin alone induced oFSHβLuc expression 11-fold, and addition of 5Z-7-Oxozeanol inhibited 100% of this induction in a dose-dependent manner, with an ED_{50} of 1.2 μM (Fig. 5A). There was no effect of 5Z-7-Oxozeanol on basal expression of oFSHβLuc (Fig. 5A).

The data in Fig. 5B are similar to those in Fig. 5A, but cotransfection of TAK1/TAB1, which constitutively activates TAK1, was used to induce oFSHβLuc instead of activin (Fig. 4A). The potency of 5Z-7-Oxozeanol was tested on LβT2 cells cotransfected with TAK1/TAB1 to compare the ED_{50} values obtained for inhibition of endogenous TAK1. Expression of oFSHβLuc was increased 35-fold by TAK1/TAB1, and 5Z-7-Oxozeanol inhibited this induction by 99% in a dose-dependent manner, with an ED_{50} of 1.5 μM. As in Fig. 5A, basal expression of oFSHβLuc was unaffected by the chemical inhibitor. These results are consistent with the concept that 5Z-7-Oxozeanol strongly inhibited the extra TAK1

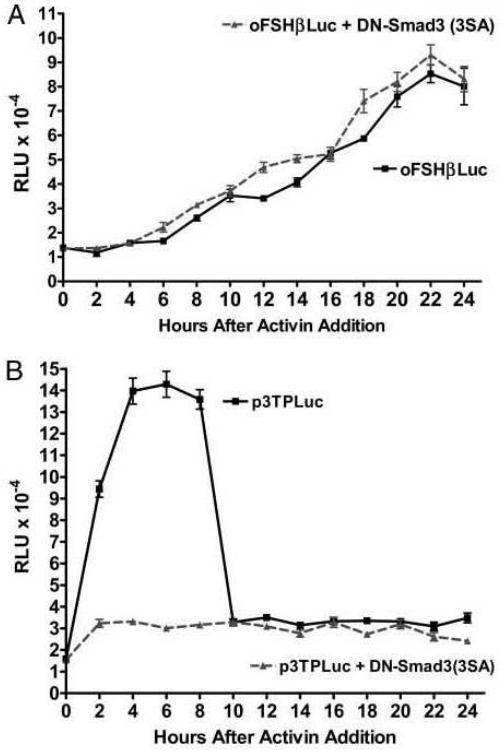


FIG. 3. Different kinetics for oFSHβLuc and p3TPLux induction by activin. LβT2 cells were prepared and plated as in Fig. 1. A, Cells were cotransfected with 50 ng oFSHβLuc plus either 50 ng pCMV (■) or 50 ng Smad3 (3SA) (▲). After transfection (24 h), triplicate cultures were sequentially treated with activin (100 ng/ml) every 2 h for 24 h. There was no significant increase in expression for the first 6 h of activin treatment for either control or DN-Smad3 (3SA)-transfected cells. Cells treated with activin for 8 h or longer showed significant increases in expression, but there was no significant difference at any time point between cultures transfected with pCMV (control) or DN-Smad3 (3SA). B, Cells were treated as in A, except p3TPLux was used instead of oFSHβLuc. All cultures treated with activin showed, at least, a significant doubling of expression at all time points ($P < 0.05$). Expression increased further ($P < 0.001$), up to 9.3-fold, between 2 and 8 h, in the absence of DN-Smad3 (3SA), but DN-Smad3 totally blocked this increase.

produced by transfecting LβT2 cells with a TAK1 expression construct.

The effects of TAK1 inhibitor 5Z-7-Oxozeanol were tested on the activin signaling pathway using Ca-ActRIB (T206D) to induce oFSHβLuc as in Fig. 2C. Cotransfection of Ca-ActRIB (T206D) stimulated oFSHβLuc by 8.4-fold (Fig. 5C), and addition of 5Z-7-Oxozeanol inhibited this expression by 95%, with an ED_{50} of 1.6 μM.

Specificity of 5Z-7-Oxozeanol inhibition

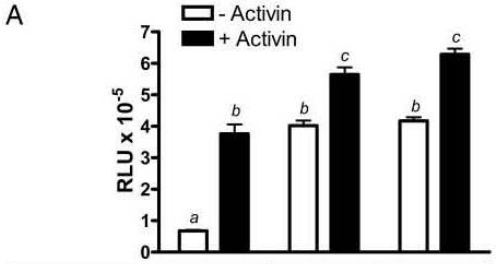
To confirm that 5Z-7-Oxozeanol was a specific inhibitor of oFSHβLuc, the effects of 5Z-7-Oxozeanol were tested on endogenous FSHβ and the two other closely related gonadotropin subunit genes expressed in LβT2 cells. LβT2 cells were treated with activin, with or without 5Z-7-Oxozeanol, and total RNA was isolated and analyzed using real-time RT-PCR for mouse FSHβ-mRNA (Fig. 6A). In addition, analyses were performed for αGSU subunit mRNA (Fig. 6B) and LHβ mRNA (Fig. 6C). The data show that 24 h of activin treatment increased mRNA for mouse FSHβ by 922-fold and that 5Z-7-Oxozeanol inhibited this induction by more than 99%. Although activin is often reported to be neutral toward expression of the αGSU, activin decreased mRNA for αGSU by 92% in this series of experiments, and 5Z-7-Oxozeanol reversed most of this inhibition. Likewise, mRNA for LHβ was slightly inhibited by activin (30% inhibition), and 5Z-7-Oxozeanol reversed this inhibition, to create an actual increase in LHβ mRNA of 60%. These data indicated that 5Z-7-Oxozeanol reversed the effects of activin on the expression of every glycoprotein hormone subunit produced in LβT2 cells. Because gene expression of αGSU and LHβ were stimulated, it was clear that 5Z-7-Oxozeanol did not inhibit protein synthesis or cellular function in general.

TAK1 is present in LβT2 cells and is phosphorylated 2–24 h after activin treatment

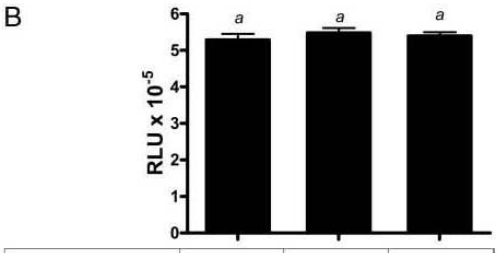
To establish the existence of TAK1 in LβT2 cells and its time-course of phosphorylation by activin, LβT2 cells were pretreated with follistatin for 16 h to bioneutralize any endogenously made activin and then treated with activin for 2–24 h. Cell extracts were prepared and analyzed by Western blot techniques to visualize TAK1, phophorylated TAK1, and TAB1 simultaneously. The data in Fig. 7 show that LβT2 cells expressed both TAK1 and TAB1 abundantly. TAK1 migrated with a molecular mass of 70 kDa, and TAB1 migrated with a molecular mass of 55 kDa, as expected (33). The immunoblot revealed that activin phosphorylated TAK1 between 1–2 h (Fig. 7). Activation of TAK1 by activin was greatest at 4, 6, and 8 h but was maintained at higher-than-control levels up to 24 h. There was no change in overall expression of TAK1 or TAB1 during activin treatment.

p38 MAPK participates in activin-mediated induction of oFSHβLuc

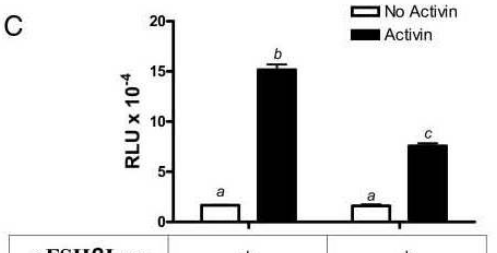
TAK1 is a member of the MAPKKK family that can phosphorylate and activate members of the MAPK family, including the ERK, JNK, and p38 MAPKs. To determine whether MAPKs are involved in activin-mediated induction of oFSHβ, cultures of LβT2 cells were treated with or without specific chemical inhibitors of ERK, JNK, and p38 MAPKs with or without activin to determine whether the inhibitors would specifically block activin induction of oFSHβLuc expression. A chemical inhibitor of JNK (JNK II inhibitor) had no effect on induction of oFSHβLuc by activin (Fig. 8A). An ERK-specific chemical inhibitor (PD98059) did not block activin induction of oFSHβLuc, but it did increase basal expression, suggesting that ERK plays an inhibitory role in basal expression of the oFSHβ-gene (Fig. 8B). Finally, a well-known inhibitor of p38 (SB203580) inhibited activin action by 60% (Fig. 8C). These results suggest that p38-MAPK is downstream of the TAK1 pathway and that activation of p38-MAPK is important for activin induction of oFSHβLuc.



oFSHβLuc	+	+	+
pCMV	+		
TAK1		+	+
TAB2		+	
TAB3			+



pGL3-Control	+	+	+
pCMV	+		
TAK1		+	+
TAB2		+	
TAB3			+



oFSHβLuc	+	+
pCMV	+	
DN-TAK1		+

FIG. 4. TAB2 or TAB3 partner with TAK1 to induce expression of oFSHβLuc like activin. LβT2 cells were prepared and plated as in Fig. 1. A, Cells were transiently cotransfected with 50 ng oFSHβLuc and 150 ng pCMV as mock DNA, or oFSHβLuc and 25 ng TAK1, and 125 ng of either TAB2 or TAB3. Twenty-four hours after transfection, cells were treated with or without activin (100 ng/ml), for an additional 24 h before analysis. *Means with different letters* are significantly different ($P < 0.05$). B, Cells were transiently cotransfected with 50 ng pGL3-control and either 150 ng mock plasmid pCMV or 25 ng TAK1 plus 125 ng TAB2 or TAB3. After transfection (24 h), cells were assayed for luciferase activity. The results of these treatments were not significantly different ($P > 0.05$). C, Cells were transiently cotransfected with 50 ng oFSHβLuc and 50 ng of either mock plasmid pCMV or expression vector encoding the TAK1 DN (DN-TAK1-KN). After 24 h of transfection, cells were treated with or without activin (100 ng/ml) for an additional 24 h. *Means with different letters* are significantly different ($P < 0.01$).

Discussion

It is well known that activin can increase the synthesis (and associated secretion) of FSH by inducing transcription of its β-subunit (FSHβ) (11, 58). It is also known that activin classically transmits its signal intracellularly by phosphorylating Smad2 or Smad3, and it was recently reported that expression of extra Smad3 (but not Smad2) increases basal and activin-induced expression of rat (30) and ovine FSHβ-subunits in LβT2 cells (29). Initial studies in our laboratory focused on the role of Smad3 as a potential mediator activated by activin; however, the data showed that activin is able to stimulate FSHβ expression independent of Smad3 activation. The studies reported here identified that a TAK1 pathway is critical for mediating activin induction of FSHβ expression in gonadotropes.

Transfection of Smad3 is known to increase basal expression of Smad-dependent genes to levels comparable with induction by activin (59). Preliminary studies showed that Smad3 overexpression stimulated both basal and activin-induced expression of the FSHβ-gene. However, overexpression of Smad3 did not increase the ratio of induction/basal expression of oFSHβLuc over a wide range of Smad3 concentrations, including those that were maximally effective. One interpretation is that overexpression of Smad3 simply stimulates basal expression of oFSHβLuc. If this is all that occurs, the increased induction by activin observed in the presence of extra Smad3 might occur simply because transcription started at a higher level. This explanation does not imply that activin works through the activation of Smad3. Therefore, the data in Fig. 1 fail to prove that Smad3 participates in activin-mediated induction of the FSHβ-subunit.

In this report, it is shown, for the first time, that inhibiting activation of endogenous Smad3 does not affect activin's ability to induce expression of the FSHβ-subunit. Two well-characterized Smad3 mutants [Smad3 (3SA) and Smad3 (D407E)] were used to block possible activation of endogenous Smad3 by activin. Smad3 (3SA) has three serines required for activation mutated to three alanines (60). Smad3 (D407E) has an aspartate mutated to glutamate, which allows it to bind tightly to the activin type I receptor's (ActRIB) active site, therefore blocking the activation of Smad2 and Smad3 signaling pathways (53). The results show that neither of these inhibitors blocked activin-induced expression of oFSHβLuc (Fig. 2, B–D). Furthermore, both DN inhibitors were shown to be fully effective in LβT2 cells, because they blocked the ability of activin to induce p3TPLuc, which is known to depend on Smad3 activation in other cell types (59). These data can only be interpreted to mean that activin does not require the activation of endogenous Smad3 to induce FSHβ-gene. Furthermore, because DN-Smad3 (D407E)

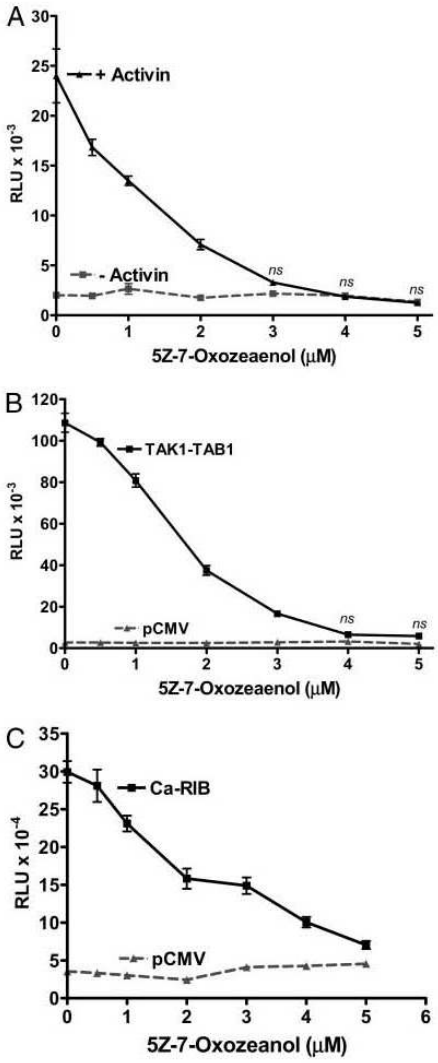


FIG. 5. Inhibition of TAK1 with 5Z-7-Oxozeanol fully blocked activin induction of oFSHβLuc expression. LβT2 cells were prepared and plated as in Fig. 1. A, Cells were transiently transfected with 50 ng oFSHβLuc promoter construct for 24 h. Cells were then treated with or without 5Z-7-Oxozeanol at the indicated concentrations for 2 h before treatment with (▲) or without activin (■) at 100 ng. Cells were incubated another 24 h before analysis for luciferase activity. Because 5Z-7-Oxozeanol is reported to be somewhat labile, halfway through the 24-h incubation, fresh media was added containing the same concentrations of 5Z-7-Oxozeanol and activin. Treatment with 5Z-7-Oxozeanol significantly inhibited induction of oFSHβLuc at all concentrations ($P < 0.05$) and completely inhibited it between 3–5 μM (ns, not significantly different from unstimulated control cultures). B, Cells were cotransfected with 50 ng oFSHβLuc and 50 ng pCMV mock DNA (▲) or 50 ng oFSHβLuc, 25 ng TAK1, and 25 ng TAB1 expression constructs (■). After transfection (24 h), cells were treated with increasing concentrations of 5Z-7-Oxozeanol as in A above. Treatment with 5Z-7-Oxozeanol significantly inhibited induction of oFSHβLuc at all concentrations ($P < 0.05$) and completely inhibited it at 4 μM and above (ns). C, Cells were transiently cotransfected with 50 ng oFSHβLuc and 50 ng of either mock plasmid pCMV (▲) or expression vector encoding the Ca-ActRIB (■). After transfection (24 h), cells were treated with increasing concentrations of 5Z-7-Oxozeanol as in A above. Treatment with 5Z-7-Oxozeanol significantly inhibited induction of oFSHβLuc at all concentrations ($P < 0.05$).

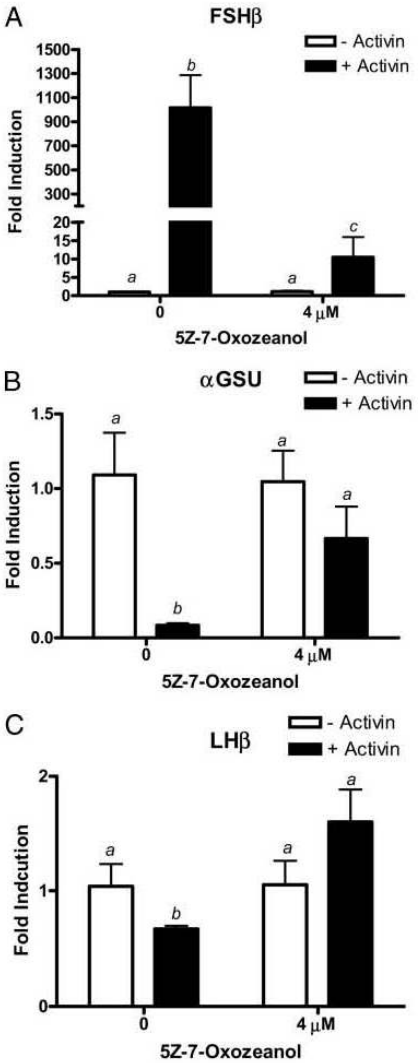


FIG. 6. LβT2 cells were plated in 6-well plates at 1 million cells per well. The cells were treated with or without activin (100 ng/ml) and treated with or without 4 μM 5Z-7-Oxozeanol. Cells were incubated for 24 h and then washed with PBS, and total RNA was extracted using Tri-Zol reagent. Threshold cycle (Ct) values for (A) FSHβ, (B) αGSU, and (C) LHβ were normalized by subtracting their respective 18s rRNA Ct values. Neither activin nor 5Z-7-Oxozeanol altered 18s rRNA levels because Ct values for 18s rRNA did not change significantly ($P > 0.05$). Normalized Ct values were averaged and used to compare FSHβ, αGSU, and LHβ in the absence or presence of activin using the $2^{-(\Delta\Delta Ct)}$ method for quantitation. The data are plotted as fold-induction of mRNA levels above basal expression. *Means with different letters* are significantly different ($P < 0.05$).

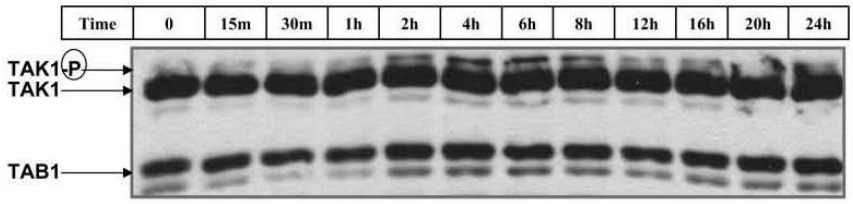


FIG. 7. Activin phosphorylated TAK1 within 2 h and maintained TAK1 activation for 24 h. LβT2 cells were plated at 1 million cells per well in 6-well plates. Cells were pretreated with follistatin-288 (250 ng/ml; 16 h) and washed with culture media. Cells were then treated with activin (100 ng/ml) for 0, 15, or 30 min or 1, 2, 4, 6, 8, 12, 16, 20, or 24 h. Phosphorylation of endogenous TAK1 was detected by Western blot analysis as described in *Materials and Methods*.

blocks activation of Smad2 along with Smad3 in other cell systems (53), its failure to block activin-mediated induction of oFSHβLuc strongly suggests that activation of neither Smad2 nor Smad3 is required for activin-mediated induction of FSHβ-subunit expression.

The results were surprising because both rat and ovine FSHβ-promoters contain consensus Smad binding sites (29, 30) at positions that are important for activin-mediated induction, and data show Smad4 binding to several of these positions. However, it should be noted that previous publications using LβT2 cells have shown Smad4 binding is observed under basal conditions and is not enhanced due to activin treatment (61). The role of Smads in binding to FSHβ-promoter elements is still unclear. It might be possible for Smads to play a role at the basal transcription complex independent of activin, where Smad3 or Smad4 are continuously moving into the nucleus to bind SBE(s) that drives basal transcription as shown in other systems (61, 62). This remains an unsolved question of activin action.

Another important finding is the different activation kinetics observed between oFSHβLuc and Smad-dependent construct (p3TPLuc) in response to activin treatment. In this report, the time-course of activin induction of Smad-dependent p3TPLuc showed very different kinetics compared with induction of oFSHβLuc (Fig. 3). Induction of p3TPLuc was relatively rapid (2–8 h), whereas induction of oFSHβLuc was observed only after 8 h of treatment. Essentially all previous reports using FSHβLuc in LβT2 cells measured activin induction at 24 h (51), which matched the optimal time observed in the studies reported here. Because Smad2 and Smad3 are normally activated within 1–4 h (49), the data in Fig. 4 provide further evidence that neither Smad2 nor Smad3 is the immediate trigger for activin induction of oFSHβLuc. Based on kinetics, however, Smad3 could be the immediate trigger for induction of p3TPLuc. These data even suggest the possibility that oFSHβLuc induction depends on one or more late genes that might be under control of one or more early response genes affected by activin, but the early genes, themselves, should not depend on Smad3 for this action because DN-Smad3 inhibitors do not block activin induction of oFSHβLuc. Multigene analysis could be necessary to study this possibility further.

The evidence showing that endogenous Smad3 (or Smad2) is not the trigger for activin-mediated induction of oFSHβLuc led to experiments that explored a Smad-independent pathway involving TAK1 (63, 64). Activin, TGFβ, and BMP were shown in multiple studies to signal through

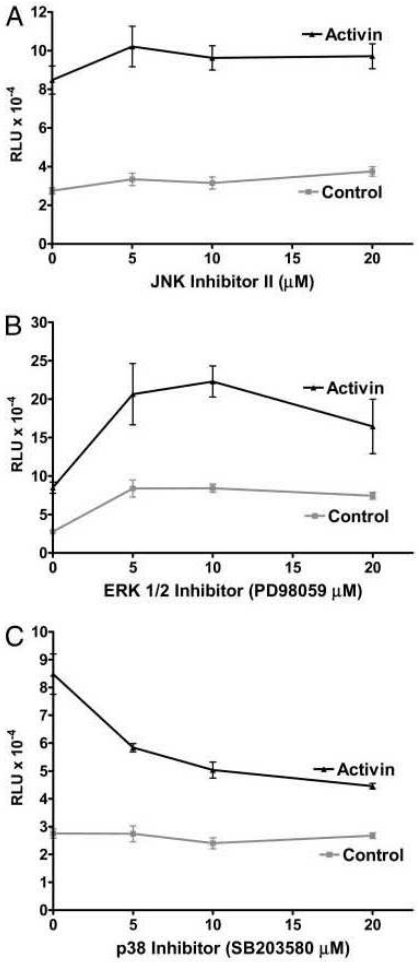


FIG. 8. Inhibition of p38-MAPK partially blocks activin induction of oFSHβ. LβT2 cells were prepared and plated as in Fig. 1A and transiently transfected with 50 ng oFSHβLuc. A, After transfection (24 h), cells were treated with or without activin (100 ng/ml) and with JNK inhibitor II (0, 5, 10, and 20 μM); B, the same as A but using the ERK inhibitor; C, the same as A but using the p38 MAPK inhibitor (0, 5, 10, and 20 μM). Cultures were treated for 24 h with or without inhibitors and then analyzed for luciferase activity. Only SB203580 significantly decreased the ratio of induction/basal expression ($P < 0.05$).

activation of TAK1 (MAPKKK), initiating a MAPK signaling cascade; therefore the initial results directed the focus of the study to the possible role of TAK1 in mediating activin stimulation of FSHβ-gene. Initially, a series of experiments with TAK1 and TAB1 showed that their dual overexpression cause robust stimulation of the oFSHβLuc, as previously shown in other systems of TAK1 (55). However, overexpression of TAK1 along with TAB1 results in constitutive kinase activation of TAK1, resulting in activating other genes including the control constructs. TAB2 and TAB3 were also identified as TAK1 binding partners, but they differ from TAK1 by their ability to bind to upstream regulatory elements of the signaling pathway. It has been recently shown that TAK1 binds TAB1 and can either partner with TAB2, forming a TAK1/TAB1/TAB2 complex, or partner with TAB3 to form a TAK1/TAB1/TAB3 complex. Therefore, experiments with TAK1 and TAB2 and TAB3 showed that overexpression of TAK1 with either TAB2 or TAB3 specifically induced oFSHβLuc. Furthermore, this induction substituted almost entirely for activin, which is consistent with TAK1 being an essential member of the activin signaling pathway. In addition, it was found that a DN inhibitor of endogenous TAK1 blocked activin-mediated induction of oFSHβLuc by 50%. This, too, was consistent with a physiological role for TAK1 in oFSHβLuc expression.

Supplementary studies with a newly available TAK1 inhibitor, 5Z-7-Oxozeanol, established that TAK1 was required for activin-mediated expression of oFSHβ. 5Z-7-Oxozeanol is a resorcylic acid lactone that is currently being investigated for its potential as a therapeutic agent for allergic cutaneous disorders. It has been shown to specifically inhibit the catalytic activity of TAK1 without affecting any of the other MAPKKK family members (55). The use of 5Z-7-Oxozeanol to inhibit endogenous TAK1 activation in LβT2 cells showed that: first, the TAK1 inhibitor was shown to fully block activin induction of oFSHβLuc in a dose-dependent manner (Fig. 5); then, it was shown that 5Z-7-Oxozeanol blocked induction of endogenous mouse FSHβ-mRNA in LβT2 cells by 92%, and specificity was established by showing that 5Z-7-Oxozeanol did not decrease mRNAs for endogenous LHβ and αGSU (Fig. 6). In fact, the TAK1 inhibitor actually increased levels of these latter mRNAs by reversing the effects of activin on these gonadotropin subunit mRNAs. Although regulation of FSHβ in LβT2 cells by activin is well established (51, 52), surprisingly activin down-regulated αGSU expression. It might be that activin regulates αGSU differently in primary and transformed LβT2 gonadotropes, or it could be that proper regulation of αGSU subunit requires paracrine factors provided by other pituitary cell types. Finally, 5Z-7-Oxozeanol fully inhibited oFSHβLuc expression in LβT2 cells cotransfected with the constitutively active activin receptor (Fig. 5B). All of these data showed that TAK1 was necessary for activin-mediated induction of the FSHβ-subunit in both the sheep and mouse in transformed LβT2 gonadotropes.

Because oFSHβLuc was induced relatively slowly by activin (after 8 h of treatment), the time-course for TAK1 phosphorylation was investigated and found to be compatible with induction of both p3TPLuc and oFSHβLuc. That is, TAK1 was abundant in LβT2 cells, and its phosphorylation was strongest from 2–8 h but continued to be elevated through 24 h. TAB1 was abundant in LβT2 cells and, presumably, TAB2 and/or TAB3 were also. Further study is needed on TAK1 activation and its relationships to its binding proteins to determine how it brings specificity of action to activin-mediated events.

Finally, TAK1 is reported to activate p38 MAPK in other signaling pathways (64). Therefore, inhibitors of ERK, JNK, and p38 MAPKs were used to determine whether any of the major MAPKs were involved in activin-mediated induction of oFSHβLuc. As expected, the inhibitor of p38 MAPK linked it with activin-mediated induction, but none of the other MAPKs were associated with activin action. These data do not prove that TAK1 is responsible for p38 activation, but the results are consistent with TAK1 activation of p38, which then activates transcription factors that directly or indirectly induce expression of the FSHβ-subunit.

In summary, it has been shown that activin stimulates induction of FSHβ through a Smad-independent pathway initiated by the activation of TAK1. Furthermore, p38 MAPK, known to be a downstream target of TAK1, was shown to be involved in activin induction of oFSHβ-gene.

Acknowledgments

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References

1. Kumar TR, Wang Y, Lu N, Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204
2. Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J 1996 Gonadotropin-releasing hormone regulates follicle-stimulating hormone-β gene expression through an activin/follistatin autocrine or paracrine loop. Endocrinology 137:3667–3673
3. Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR, Marshall JC 1989 The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. Endocrinology 125:917–924
4. Haisenleder DJ, Ortolano GA, Dalkin AC, Ellis TR, Paul SJ, Marshall JC 1990 Differential regulation of gonadotropin subunit gene expression by gonadotropin-releasing hormone pulse amplitude in female rats. Endocrinology 127: 2869–2875
5. Huang HJ, Wu JC, Su P, Zhirnov O, Miller WL 2001 A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology 142:2275–2283
6. Krummen LA, Woodruff TK, DeGuzman G, Cox ET, Baly DL, Mann E, Garg S, Wong WL, Cossum P, Mather JP 1993 Identification and characterization of binding proteins for inhibin and activin in human serum and follicular fluids. Endocrinology 132:431–443
7. Ling N, Ying SY, Ueno N, Esch F, Denoroy L, Guillemin R 1985 Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217–7221
8. Otsuka F, Shimasaki S 2002 A novel function of bone morphogenetic pro-

tein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 143:4938–4941
9. Rivier J, Spiess J, McClintock R, Vaughan J, Vale W 1985 Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Commun 133:120–127
10. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J 1986 Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 321:776–779
11. Carroll RS, Corrigan AZ, Gharib SD, Vale W, Chin WW 1989 Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. Mol Endocrinol 3:1969–1976
12. Norwitz ER, Xu S, Jeong KH, Bedecarrats GY, Winebrenner LD, Chin WW, Kaiser UB 2002 Activin A augments GnRH-mediated transcriptional activation of the mouse GnRH receptor gene. Endocrinology 143:985–997
13. Norwitz ER, Xu S, Xu J, Spiryda LB, Park JS, Jeong KH, McGee EA, Kaiser UB 2002 Direct binding of AP-1 (Fos/Jun) proteins to a SMAD binding element facilitates both gonadotropin-releasing hormone (GnRH)- and activin-mediated transcriptional activation of the mouse GnRH receptor gene. J Biol Chem 277:37469–37478
14. Attisano L, Wrana JL, Cheifetz S, Massague J 1992 Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell 68:97–108
15. Mathews LS, Vale WW 1991 Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65:973–982
16. ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K 1993 Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8:2879–2887
17. Attisano L, Carcamo J, Ventura F, Weis FM, Massague J, Wrana JL 1993 Identification of human activin and TGFβ type I receptors that form heteromeric kinase complexes with type II receptors. Cell 75:671–680
18. Attisano L, Wrana JL, Montalvo E, Massague J 1996 Activation of signalling by the activin receptor complex. Mol Cell Biol 16:1066–1073
19. Carcamo J, Weis FM, Ventura F, Wieser R, Wrana JL, Attisano L, Massague J 1994 Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor β and activin. Mol Cell Biol 14:3810–3821
20. Mathews LS, Vale WW, Kintner CR 1992 Cloning of a second type of activin receptor and functional characterization in Xenopus embryos. Science 255:1702–1705
21. Chen X, Rubock MJ, Whitman M 1996 A transcriptional partner for MAD proteins in TGF-β signalling. Nature 383:691–696
22. Hua X, Liu X, Ansari DO, Lodish HF 1998 Synergistic cooperation of TFE3 and smad proteins in TGF-β-induced transcription of the plasminogen activator inhibitor-1 gene. Genes Dev 12:3084–3095
23. Labbe E, Silvestri C, Hoodless PA, Wrana JL, Attisano L 1998 Smad2 and Smad3 positively and negatively regulate TGFβ-dependent transcription through the forkhead DNA-binding protein FAST2. Mol Cell 2:109–120
24. Massague J 1998 TGF-β signal transduction. Annu Rev Biochem 67:753–791
25. Pouponnot C, Jayaraman L, Massague J 1998 Physical and functional interaction of SMADs and p300/CBP. J Biol Chem 273:22865–22868
26. Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP 1998 Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-β signaling. Cell 94:585–594
27. Wotton D, Lo RS, Lee S, Massague J 1999 A Smad transcriptional corepressor. Cell 97:29–39
28. Zhou S, Zawel L, Lengauer C, Kinzler KW, Vogelstein B 1998 Characterization of human FAST-1, a TGFβ and activin signal transducer. Mol Cell 2:121–127
29. Bailey JS, Rave-Harel N, McGillivray SM, Coss D, Mellon PL 2004 Activin regulation of the follicle-stimulating hormone β-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol Endocrinol 18:1158–1170
30. Suszko MI, Lo DJ, Suh H, Camper SA, Woodruff TK 2003 Regulation of the rat follicle-stimulating hormone β-subunit promoter by activin. Mol Endocrinol 17:318–332
31. Gregory SJ, Lacza CT, Detz AA, Xu S, Petrillo LA, Kaiser UB 2005 Synergy between activin A and gonadotropin-releasing hormone in transcriptional activation of the rat follicle-stimulating hormone-β gene. Mol Endocrinol 19:237–254
32. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K 1995 Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science 270:2008–2011
33. Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K 1996 TAB1: an activator of the TAK1 MAPKKK in TGF-β signal transduction. Science 272:1179–1182
34. Cheung PC, Nebreda AR, Cohen P 2004 TAB3, a new binding partner of the protein kinase TAK1. Biochem J 378:27–34
35. Jin G, Klika A, Callahan M, Faga B, Danzig J, Jiang Z, Li X, Stark GR, Harrington J, Sherf B 2004 Identification of a human NF-κB-activating protein, TAB3. Proc Natl Acad Sci USA 101:2028–2033
36. Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ 2004 TAB2 and TAB3 activate the NF-κB pathway through binding to polyubiquitin chains. Mol Cell 15:535–548
37. Ishitani T, Takaesu G, Ninomiya-Tsuji J, Shibuya H, Gaynor RB, Matsumoto K 2003 Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. EMBO J 22:6277–6288
38. Munoz-Sanjuan I, Bell E, Altmann CR, Vonica A, Brivanlou AH 2002 Gene profiling during neural induction in *Xenopus laevis*: regulation of BMP signaling by post-transcriptional mechanisms and TAB3, a novel TAK1-binding protein. Development 129:5529–5540
39. Qiao B, Padilla SR, Benya PD 2005 TGF-β activated kinase 1 (TAK1) mimics and mediates TGF-β-induced stimulation of type II collagen synthesis in chondrocytes independent of Col2a1 transcription and Smad3 signaling. J Biol Chem
40. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K, Nishida E 1999 Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-β-induced gene expression. J Biol Chem 274:27161–27167
41. Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Maekawa T, Ishii S 1999 ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-β signaling. J Biol Chem 274:8949–8957
42. Schnabl B, Bradham CA, Bennett BL, Manning AM, Stefanovic B, Brenner DA 2001 TAK1/JNK and p38 have opposite effects on rat hepatic stellate cells. Hepatology 34:953–963
43. Akiyama S, Yonezawa T, Kudo TA, Li MG, Wang H, Ito M, Yoshioka K, Ninomiya-Tsuji J, Matsumoto K, Kanamaru R, Tamura S, Kobayashi T 2004 Activation mechanism of c-Jun amino-terminal kinase in the course of neural differentiation of P19 embryonic carcinoma cells. J Biol Chem 279:36616–36620
44. Goswami M, Uzgare AR, Sater AK 2001 Regulation of MAP kinase by the BMP-4/TAK1 pathway in Xenopus ectoderm. Dev Biol 236:259–270
45. Dowdy SC, Mariani A, Janknecht R 2003 HER2/Neu- and TAK1-mediated up-regulation of the transforming growth factor β inhibitor Smad7 via the ETS protein ER81. J Biol Chem 278:44377–44384
46. Sakurai H, Nishi A, Sato N, Mizukami J, Miyoshi H, Sugita T 2002 TAK1-TAB1 fusion protein: a novel constitutively active mitogen-activated protein kinase kinase kinase that stimulates AP-1 and NF-κB signaling pathways. Biochem Biophys Res Commun 297:1277–1281
47. Chen Y, Lebrun JJ, Vale W 1996 Regulation of transforming growth factor β- and activin-induced transcription by mammalian Mad proteins. Proc Natl Acad Sci USA 93:12992–12997
48. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF 1999 Targeted disruption of Smad3 reveals an essential role in transforming growth factor β-mediated signal transduction. Mol Cell Biol 19:2495–2504
49. Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT, Wang XF 1997 Tumor suppressor Smad4 is a transforming growth factor β-inducible DNA binding protein. Mol Cell Biol 17:7019–7028
50. Wagner M, Kleeff J, Lopez ME, Bockman I, Massaque J, Korc M 1998 Transfection of the type I TGF-β receptor restores TGF-β responsiveness in pancreatic cancer. Int J Cancer 78:255–260
51. Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL 2001 Cell-specific transcriptional regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell model. Endocrinology 142:2284–2295
52. Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL 2001 The promoter for the ovine follicle-stimulating hormone-β gene (FSHβ) confers FSHβ-like expression on luciferase in transgenic mice: regulatory studies *in vivo* and *in vitro*. Endocrinology 142:2260–2266
53. Goto D, Yagi K, Inoue H, Iwamoto I, Kawabata M, Miyazono K, Kato M 1998 A single missense mutant of Smad3 inhibits activation of both Smad2 and Smad3, and has a dominant negative effect on TGF-β signals. FEBS Lett 430:201–204
54. Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA 2002 Transforming growth factor-β stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. J Biol Chem 277:24571–24578
55. Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, Mihara M, Tsuchiya M, Matsumoto K 2003 A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. J Biol Chem 278:18485–18490
56. Ono K, Ohtomo T, Ninomiya-Tsuji J, Tsuchiya M 2003 A dominant negative TAK1 inhibits cellular fibrotic responses induced by TGF-β. Biochem Biophys Res Commun 307:332–337
57. Wu JC, Su P, Safwat NW, Sebastian J, Miller WL 2004 Rapid, efficient isolation of murine gonadotropes and their use in revealing control of follicle-stimulating hormone by paracrine pituitary factors. Endocrinology 145:5832–5839
58. Weiss J, Guendner MJ, Halvorson LM, Jameson JL 1995 Transcriptional activation of the follicle-stimulating hormone β-subunit gene by activin. Endocrinology 136:1885–1891
59. Wang EY, Ma EY, Woodruff TK 2003 Activin signal transduction in the fetal rat adrenal gland and in human H295R cells. J Endocrinol 178:137–148

60. Liu X, Sun Y, Constantinescu SN, Karam E, Weinberg RA, Lodish HF 1997 Transforming growth factor β-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. Proc Natl Acad Sci USA 94:10669–10674
61. Liu C, Gaca MD, Swenson ES, Vellucci VF, Reiss M, Wells RG 2003 Smads 2 and 3 are differentially activated by transforming growth factor-β (TGF-β) in quiescent and activated hepatic stellate cells. Constitutive nuclear localization of Smads in activated cells is TGF-β-independent. J Biol Chem 278:11721–11728
62. Inagaki Y, Mamura M, Kanamaru Y, Greenwel P, Nemoto T, Takehara K, Ten Dijke P, Nakao A 2001 Constitutive phosphorylation and nuclear localization of Smad3 are correlated with increased collagen gene transcription in activated hepatic stellate cells. J Cell Physiol 187:117–123
63. Zhang L, Deng M, Parthasarathy R, Wang L, Mongan M, Molkentin JD, Zheng Y, Xia Y 2005 MEKK1 transduces activin signals in keratinocytes to induce actin stress fiber formation and migration. Mol Cell Biol 25:60–65
64. Ogihara T, Watada H, Kanno R, Ikeda F, Nomiyama T, Tanaka Y, Nakao A, German MS, Kojima I, Kawamori R 2003 p38 MAPK is involved in activin A- and hepatocyte growth factor-mediated expression of pro-endocrine gene neurogenin 3 in AR42J-B13 cells. J Biol Chem 278:21693–21700

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CHAPTER III

Expression and regulation of the β-subunit of ovine follicle-stimulating hormone relies heavily on a promoter sequence likely to bind Smad-associated proteins

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Expression and Regulation of the β-Subunit of Ovine Follicle-Stimulating Hormone Relies Heavily on a Promoter Sequence Likely to Bind Smad-Associated Proteins

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FSH is essential for normal gonadal function in mammals. Expression of its β-subunit (FSHB) controls overall production/secretion of FSH and is induced by activin. Studies with ovine *FSHB* promoter/reporter constructs in LβT2 gonadotropes show that induction by activin requires a putative Smad binding element in the ovine *FSHB* promoter (-162AGAC-159). Similar studies reported here show that another site, juxtaposed to the Smad binding element, was also required for 81% activin induction in LβT2 cells. This site was similar to several that bind proteins known to partner with Smads. When this site (-171ACTgcgtTT-163) was mutated by changing the nucleotides shown in lowercase letters, the resulting ovine-derived construct (mut-o*FSHBLuc*) was expressed poorly as a transgene in primary mouse gonadotropes (<0.001 times compared with ovine wild-type transgenes). This decrease in expression demonstrated the importance of this site for activin induction and, perhaps, basal expression, although studies with LβT2 cells did not suggest this latter possibility. Expression of mut-o*FSHBLuc* in male mouse gonadotropes *in vivo* was at least 644 times greater than expression in all but one nongonadotrope tissue tested, indicating that mut-o*FSHBLuc* retained significant gonadotrope-specific expression. An increase in FSHB expression occurs during estrus in mice and is faithfully reproduced with wild-type ovine *FSHBLuc* transgenes, but not with mut-o*FSHBLuc*, indicating that the mutated site is needed for this secondary FSH surge. These data suggest that activin gathers Smads and Smad-associated proteins at the −171/−159 promoter region to regulate expression of the ovine FSHB and overall FSH production. (*Endocrinology* 148: 4500–4508, 2007)

FSH IS ESSENTIAL for ovarian follicular development, and enhances the maturation and performance of sperm (1). Because of its importance, FSH is carefully regulated by more than six reproductive hormones that ensure its correct production and secretion. One of these hormones, activin, is recognized as an important inducer of FSH. Studies by others show that follistatin and inhibin (known inhibitors of activin action) decrease FSH production by 50% in primary rat pituitary cultures (2) and lower serum levels of FSH in ovariectomized rats by 50–60% within 4–5 h (3). More recent data show that mice lacking type II activin receptors produce significantly less FSH than normal (~34% normal males) (4). In an attempt to learn more about the molecular mechanism(s) used by activin at the gene level, studies have recently focused on transcriptional regulation of the FSH β-subunit (FSHB), which is rate limiting for overall FSH production.

Ovine *FSHB* promoter/reporter constructs (o*FSHBLuc*; 4.7 kb of the ovine *FSHB* promoter plus intron 1 attached to the luciferase gene) are expressed in LβT2 transformed gonadotropes in ways that permit identification of promoter elements associated with induction by activin. Because Smads are recognized activin-activated transcription factors, putative Smad binding elements (SBEs) were sought and found in the proximal promoter of ovine *FSHB*. Destruction of the putative SBE at −162 bp (AGAC) is especially effective in blocking activin induction of reporter genes driven by the ovine *FSHB* promoter (5).

Similar studies in our laboratory, reported here, had discovered sequences adjacent to the −162-bp consensus Smad binding site that were also important for ovine FSHB expression in LβT2 cells in culture (6). This DNA sequence was recently identified by computer analysis to be a Runx1 binding site (7). This correlation is significant because Runx family members are known to bind the same sequence and interact with Smads through their Runt homology domains (8, 9). However, the correlation does not prove that any Runx family member binds this sequence. In fact, the DNA sequence of interest also resembles binding sites for forkhead proteins such as FAST-1 (FoxH1), a well-characterized DNA binding partner of Smads 2 and 3 that mediates many actions of TGFβ1 and activin (9, 10). Therefore, it is possible that forkhead transcription factors may, in fact, act through the putative Runx1 binding site to partner with Smads for the induction of FSHB in gonadotropes. The important point is that the sequence of interest in this report is associated with transcription factors known to partner with Smads.

To date, no laboratory using methods such as EMSA has been able to demonstrate that activin promotes the association of any protein to either the putative Runx1 or SBE

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Abbreviations: ECM, Estrous Cycle Monitor; FSHB, FSH β-subunit; RLU, relative light unit; SBE, Smad binding element.

binding site. Therefore, both binding sites remain "putative" and will be referred to this way herein because their abilities to bind these proteins have not been established. In addition, the importance of these sites has been established only in LβT2 cells. Thus, it is important to show the physiological relevance of this promoter region for FSHB expression and activin induction *in vivo*. The studies described here were designed to demonstrate the importance of this novel sequence *in vitro* and also in transgenic mice *in vivo*.

Materials and Methods

Hormones (activin A, follistatin-288, and D-Lys^6-GnRH)

Recombinant human follistatin-288 was provided by the National Pituitary and Hormone Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Recombinant human activin A was obtained from R&D Systems, Inc. (Minneapolis, MN) and was dissolved in PBS containing 0.1% serum albumin. [D-Lys^6]-GnRH (referred to as GnRH in this text) was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in 0.01 M acetic acid before use.

Universal RIA for FSH (pan-FSH RIA)

Sheep and mouse FSH were measured using a pan-FSH RIA distributed by the National Institute of Diabetes and Digestive and Kidney Diseases using a double-antibody method as previously reported (11). Purified mouse FSH (AFP-5308D) was used as tracer and standard for the mouse assay, and 95% pure ovine FSH standard was used for the sheep assay. Intraassay variation for each RIA was less than 10%, and assay slopes were more than one.

Plasmid constructs

Our wt-o*FSHBLuc*, reported in 2001 (11), was used in this study to transiently transfect LβT2 cells and also to produce all the other constructs in this study. This wild-type construct contains 4.7-kb ovine *FSHB* promoter plus intron 1 driving luciferase expression. The constructs used in Fig. 2 were made from wt-o*FSHBLuc* by producing point mutations that created novel restriction sites: constructs 1, 4, 5, 6, 9, 10, 11, and 12 contained distinguishing *Bgl*2 sites; construct 2 had a novel *Mlu*1 site; construct 3 had an *Xho*1 site; and construct 8 had a new *Eco*R1 site. All plasmids were sequenced and shown to contain the sequences depicted in Fig. 2 (SeqWright, Houston, TX). Construct 3 contained a mutation that destroyed the putative Runx1 enhancer located between −171 and −163 bp of the ovine *FSHB* promoter. This construct was transiently expressed in LβT2 cells and also was used as a transgene in mice. The transgene made from construct No. 3 was named mut-o*FSHBLuc* in this report.

Our laboratory has produced and reported on 12 transgenic mouse lines that contained the intact wild-type promoter (11, 12), but these mice were all terminated by the time this study began. These former transgenic mouse lines were replaced by seven new lines that expressed luciferase as well as the original wild-type transgenes. These new lines contained distal 5′ deletions (deletions 5′ to −2817 bp), but all expressed luciferase in the same range as the original wild-type transgenic mice. Furthermore, luciferase expression was also regulated by activin in a similar manner. One of these lines (Lj) was chosen as the wild-type "standard" for this study because it produced average amounts of luciferase activity (average luciferase activity with regard to all previous wild-type transgenic lines) and was regulated by activin in a representative manner. The wtLj-o*FSHBLuc* transgene that was used in this study lacked sequences from −4736 to −3980 bp and −3398 to −2817 bp.

All the transgenes used or referred to in this study were cut from their parent plasmid by digestion with BamH1/KpnI, which left less than 100-bp plasmid sequence on each end of the transgene. As with all previous transgenes produced in this laboratory [wt-o*FSHBLuc* (11) and mut-o*FSHBLuc*-ΔAP1 (12)], wtLj-o*FSHBLuc* and mut-o*FSHBLuc* reported here were expressed in every founder line that carried the respective transgene.

Animal care: sheep and transgenic mice

Ewes and transgenic mice were maintained and studied with the approval and oversight of the Institutional Animal Care and Use Committee at the University of North Carolina Chapel Hill or North Carolina State University. Mice containing wild-type (wtLj-o*FSHBLuc*) or mutant (mut-o*FSHBLuc*) transgenes were produced in B6SJL mice as described earlier (11), but at the transgenic mouse facility of the University of North Carolina Chapel Hill. Mice were bred and cared for at the Biological Resource Facility of North Carolina State University. Retired breeder ewes were kept at a North Carolina State University farm. Testing mice for the presence of a transgene and measuring luciferase activity in tissues was performed as previously reported (11, 12).

Cell cultures

Primary cells from ewe pituitaries were dispersed and cultured in 24-multiwell tissue culture plates with 200,000 cells per well, as described previously (13). Primary cell cultures from mouse pituitaries were also prepared as described (11, 12) and incubated in 96-well tissue culture plates at 30,000–60,000 cells per well in media 199 plus 10% charcoal-treated sheep serum. Cells were allowed to attach and adjust to culture conditions for at least 1 d before treatment. Purified gonadotropes were obtained from 8-wk-old crossbred male mice hemizygous for wt-o*FSHB-H2K*^{k} (14) and mut-o*FSHBLuc*. Purification was as described (14), except the biotin anti-H-2K^k antibody (BD PharMingen, San Diego, CA) was doubled to increase gonadotrope yield to approximately 16,000 per pituitary (>90% recovery of 95% pure gonadotropes).

Luciferase data in Fig. 5 were obtained from cultures treated on the afternoon of d 2 with or without 250 ng/ml follistatin for 16 h. Media containing follistatin were removed on the morning of d 3, cultures were washed once with fresh media, and then incubated in medium 199 with 1% serum instead of 10% serum (to restrain autocrine/paracrine production of activin) and the following hormones: follistatin (250 ng/ml), activin (50 ng/ml), GnRH (1 nM), or activin plus GnRH. After 6 h, cells were assayed for luciferase activity.

Primary sheep pituitary cultures are known to express robustly FSH for weeks without a decline in production (15), so studies with ewe cultures extended over 5 d. By contrast, mouse pituitary cultures express FSH best on d 2 and 3, after which FSH expression declines sharply and unpredictably (11). Thus, experiments with mouse cultures were completed during the d 2–3 timeframe after pituitary dispersion. Either female or male mouse pituitaries were used because preliminary studies showed that there was no sex difference in response to activin, follistatin, or GnRH in tissue culture (data not shown).

Transformed gonadotropes (LβT2 cells) were cultured at 37 C in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (HyClone Laboratories, Inc., Logan, UT) and plated at a density of 30,000 cells per well in 96-well culture plates. Cells were transfected 24 h later in triplicate or quadruplicate with 50 ng plasmid as described (16). Briefly, 50 ng wild-type or mutant ovine *FSHB* plasmids were transfected into each well. Cells were also cotransfected with 5 ng/well pRL-TK, a plasmid used to express *Renilla* as an internal standard for transient expression assays (Promega Corp., Madison, WI). Transfections were performed with Fugene 6 (Roche, Indianapolis, IN) using 165 ng/well. Plasmids and Fugene 6 were incubated in serum-free DMEM for 15 min at RT, and then 50 μl was added to each well. After 24 h, cells were treated with or without 50 ng/ml activin for 22 h and then assayed for luciferase activity.

Induction of transgenes during the reproductive cycle

Female mice (4 months old) were housed in cages containing male bedding for 2 wk before testing to promote estrous activity. Littermates (six to eight) were paired with each other for these studies with equal numbers being assigned to either diestrus or estrus groups; with odd-numbered litters, the extra mouse was assigned to the estrus group. Reproductive stages were determined using an Estrous Cycle Monitor (ECM) (Model EC40; Fine Science Tools, Foster City, CA), followed by cytological inspection of vaginal exfoliative cells as reported earlier (11, 12). Diestrus was characterized by an ECM reading of 2.5–3.5 and a preponderance of small round leukocytes. Estrus was characterized by an ECM reading of 9–11 and the presence of large nucleated cells plus

some leukocytes. After determining the reproductive status of each mouse (between 0900 and 1100 h), pituitaries were immediately removed, homogenized, and assayed for luciferase activity and protein content as described earlier (11, 12). Data from littermates were compared to determine "fold-induction at estrus."

Firefly and Renilla luciferase assays

Firefly luciferase was measured to detect expression of the wtLj-o*FSHBLuc* or mut-o*FSHBLuc* transgenes in mouse pituitary cultures or mouse tissues. For primary pituitary cultures, cells were lysed in 96-well plates with 50 μl Passive Lysis solution (Promega Corp.), followed by analyzing 35 μl with a Luciferase Assay System (Promega Corp.). Luminescence for data in Table 1 and Fig. 3 was obtained using a Monolight 2000 as previously reported (11, 12, 16), and data are reported as relative light units (RLUs). LβT2 cells (Figs. 2, 4, and 5) were lysed with 25 μl Passive Lysis Buffer (Promega Corp.), and 10 μl was assayed for luciferase activity using an automated 1420 Victor-Light micro plate luminometer (PerkinElmer, Waltham, MA). The Victor-Light luminometer was five times more sensitive than the Monolight luminometer, but all experiments in this study were done with either one or the other, and no comparisons were necessary between instruments.

Firefly luciferase was measured in whole tissue (pituitary, liver, gonads, spleen, lung, forebrain) that was excised within 5 min of death and immediately frozen in liquid nitrogen until the assay, which occurred within 24 h of tissue extraction. Pituitary tissue (~1 mg wet weight) and nonpituitary tissue (~30 mg) were homogenized in 100 μl Passive Lysis buffer, and 20 μl of each sample was assayed in duplicate. Protein concentrations were determined using bicinchoninic acid (Pierce Chemical Co., Rockford, IL).

Renilla luciferase expression was used as an internal standard when the activities of mutant ovine *FSHβLuc* constructs were compared in LβT2 cells. The *Renilla* construct (pRL-TK; 5 ng/well) was cotransfected with 50 ng/well of ovine *FSHβLuc* construct (Fig. 2). Both Firefly and *Renilla* luciferase activities were sequentially measured in the same samples using the Dual-Luciferase Assay System (Promega Corp.). For the data in Fig. 2, LβT2 cells in 96-well plates were lysed with 25 μl Passive Lysis solution, followed by analysis of 10 μl lysate.

Quantifying transgene copy number per cell

Transgene copy number was quantified in transgenic mouse lines using real-time PCR performed in an iCycler (Bio-Rad, Inc., Hercules, CA) with TaqMan technology. The luciferase gene was quantified, and 11 luciferase standards were prepared (0.016–17 pg/μl) using our pGL3-based original wild-type ovine *FSHBLuc* plasmid (10,345 bp) (11). This plasmid was quantified using a Qubit Fluorometer (Invitrogen, Eugene, OR) following their recommended protocol and Quant-iT dsDNA BR assay kit (Invitrogen). Samples were then diluted in distilled water containing 10 μg/ml salmon sperm DNA as carrier (Invitrogen). Two microliters of DNA were assayed per sample starting at 95 C for 3 min, and then for 40 complete cycles (95 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec). There was a final extension step of 72 C for 3 min. Threshold cycle values were determined with Bio-Rad software and plotted *vs.* log of plasmid DNA, and ultimately *vs.* log of copy number based on the amount of chromosomal DNA assayed per sample (20 ng). The standard curve had a threshold cycle axis with values from 19–30 and an x-axis from 0.1–3.0 genomic copies per cell ($r^2 = 0.999$). The forward oligonucleotide was 5′-GAACTGTGTGTGAGAGGTCCTATG-3′, the reverse oligonucleotide was 5′-GCTATGTCTCCAGAATGTAGC-3′, and the FAM probe was 5′ACCAACGCCTTGATTGACAAG3′. All oligonucleotides were complementary to sequences within the luciferase portion of the transgene.

Statistical analysis

Statistical calculations were performed using Prism version 4 (GraphPad software, Inc., San Diego, CA). The Student's *t* test was used when comparing two means. When more than two means were compared, one-way ANOVA was used with Tukey's multiple comparison test. Data are reported as means ± SEM throughout the paper.

Results

Expression of FSH and its inhibition by follistatin in ovine pituitary cultures

The data in Fig. 1 show that FSH was expressed in primary ovine pituitary cultures and that follistatin (25, 75, 225, and 675 ng/ml) blocked FSH production with an IC_{50} near 20 ng/ml during the first 24 h. By d 2, 225 and 675 ng/ml inhibited FSH production by 95% and 99%, respectively. These data created a normal saturation curve predicted by an IC_{50} of approximately 20 ng/ml. Ovine cultures were maintained 5 d, and the media were collected every 24 h with new media replacing the old. Therefore, each FSH value represented hormone that had accumulated during the previous 24-h period. Because activin action is not halted immediately by follistatin (there is typically a 4-h delay before inhibition by either inhibin or follistatin; Miller, W. L., unpublished data), the small amount of FSH observed after 1-d treatment with 225 or 675 ng/ml follistatin (~14% compared with control) was probably secreted before follistatin became fully effective against activin action.

Activin induction of wild-type and mutant ovine FSHBLuc constructs in LβT2 cells

The data in Fig. 2 show that expression of wt-o*FSHBLuc* increased 7.1-fold after a 22-h treatment with activin in LβT2 cells (Fig. 2, *wt*). Activin also routinely increased the expression of *Renilla* by 50–75% (data not shown), and all luciferase data were corrected for alterations in expression of *Renilla* with or without activin. Mutations from −174 to −143 bp revealed an activin sensitive region between −168 and −157 bp. Mutations at the edges of this region (constructs 1, 2, 10, 11, and 12) all showed induction of 6.9 ± 0.3, which was not different from wild-type induction. Mutations within the

TABLE 1. Luciferase activity in mut-o*FSHBLuc* transgenic mouse tissues

Founder	Sex	Pituitary	Liver	Gonad	Spleen	Lung	Brain
M248	M (5)	28 ± 3	1.0 ± 0.10	0.8 ± 0.04	0.1 ± 0.06	0.3 ± 0.04	7.5 ± 1.1
	F (5)	38 ± 2	0.6 ± 0.01	1.0 ± 0.05	ND	ND	8.4 ± 0.05
M157	M (5)	8 ± 1	0.33 ± 0.01	0.31 ± 0.02	0.07 ± 0.04	0.16 ± 0.02	1.30 ± 0.04
	F (5)	6.4 ± 0.6	0.19 ± 0.01	0.22 ± 0.01	ND	ND	1.02 ± 0.11
M230	M (4)	5.2 ± 0.2	0.23 ± 0.02	0.08 ± 0.00	ND	ND	0.42 ± 0.02
	F (5)	7.1 ± 0.4	0.21 ± 0.02	0.11 ± 0.01	ND	ND	0.72 ± 0.04
M251	M (6)	5.8 ± 0.8	0.18 ± 0.03	0.18 ± 0.02	ND	ND	0.93 ± 0.13
	F (6)	6.2 ± 0.4	0.15 ± 0.01	0.15 ± 0.01	ND	ND	0.83 ± 0.05

Expression of the mut-o*FSHBLuc* transgene in tissues. Tissues were harvested from mice between 7 and 20 weeks old, and lysates were assayed for luciferase activity. Values are luciferase activity expressed as RLUs × 10^{-3}/mg protein. The number of animals used is designated in *parentheses* according to "sex," and values represent the mean ± SEM for each group of mice. M, Male; F, female; ND, not determined.

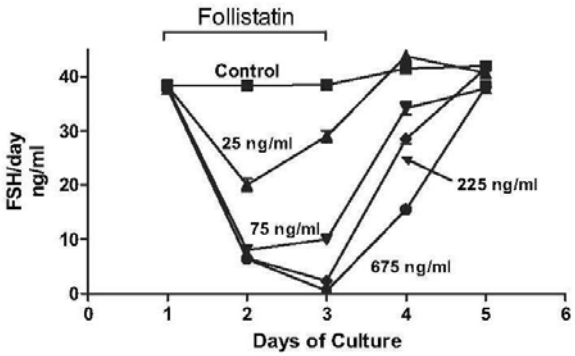

FIG. 1. Follistatin inhibited FSH production in primary ovine pituitary cultures. Ewe pituitary cultures were prepared and treated with 25, 75, 225, or 675 ng/ml follistatin on d 1, and media were collected for RIA every 24 h thereafter through d 5. Follistatin treatment ended on d 3, after which cultures were washed once with fresh media, and then new media were added and assayed/changed every 24 h as previously described. Inhibition was 99 ± 1% on d 3 with 675 ng/ml follistatin. Expression from all treatments was statistically the same on d 5. To avoid normalizing data, results are the mean ± SEM from a representative sheep (ewe) pituitary culture, and each data point was obtained from triplicate treatments each assayed in duplicate.

sensitive region (constructs 3–9) showed an average induction of only 2.2 ± 0.1. Thus, mutations within the sensitive region reduced induction by 68 ± 6%. Statistically, there was no difference among the responses of the following fully responsive constructs: 1, 2, 10, 11, and 12. Likewise, there were no statistical differences among the responses of constructs that showed reduced induction by activin (constructs 3–9). There were no significant changes in the basal expression of any construct compared with wild-type basal expression.

Low expression of mut-oFSHBLuc (construct 3 in Fig. 2) in transgenic mice

Figure 3 shows the specific activities of luciferase expression in male pituitaries from 10 transgenic mouse lines that expressed mut-o*FSHBLuc* in a hemizygous manner. Luciferase expression for all founder lines in Fig. 3 (except M248 and M231) averaged $9.0 \times 10^3 \pm 3 \times 10^3$ RLU/mg protein. Expression for M248 and M231 was $34 \times 10^3 \pm 2 \times 10^3$ RLU/mg protein and $99 \times 10^3 \pm 8 \times 10^3$RLU/mg protein, respectively. Because several transgenes can insert into a single chromosomal locus, the number of transgenes present in each cell for each transgenic line was quantified. All mice shown in Fig. 3 contained 12–23 copies of the transgene per cell except M231, which contained 283 copies per cell (data not shown). Two transgenic lines were excluded because they contained ≥3000 copies of the transgene, although these two lines did not express luciferase significantly differently from the average low expression of 9×10^3 RLU/mg protein.

Pituitary expression of the wild-type transgene, wtLj-o*FSHBLuc*, was $13{,}100 \times 10^3 \pm 120 \times 10^3$ RLU/mg protein, which is nearly 1500 times greater than the average of all mut-o*FSHBLuc* lines in Fig. 3, excluding M248 and M231. The wild-type mice expressing wtLj-o*FSHBLuc* contained only two transgene copies per cell. Finally, it should be noted that even the highest expressing mutant line (M231) did not express mut-o*FSHBLuc* at a level higher than 0.7% of the wild-type construct, wtLj-o*FSHBLuc*.

Tissue-specific expression of mut-oFSHBLuc in transgenic mice

Table 1 shows luciferase expression in the pituitaries, livers, gonads (testis and ovaries), and forebrain of mice from four founder lines harboring mut-o*FSHBLuc*. Luciferase activities were also determined for the spleen and lung in two of these lines (M248 and M157). In essentially all cases, expression of the transgene in nonpituitary tissue was ≤ 3% of that found in pituitary tissue, except for the forebrain region, where expression averaged 16% of that found in the pituitary.

Our laboratory previously showed that the wt-o*FSHBLuc* transgene is expressed almost exclusively in pituitary gonadotropes, which comprise only 3–5% the male mouse pituitary (14). To determine gonadotrope-specific expression of mut-o*FSHBLuc*, only gonadotropes were isolated from male transgenic mice (M248 line only), and their expression was compared with that of unpurified dispersed pituitary cells in the presence of 50 ng/ml activin (same conditions as for wild-type in Ref. 14). In three independent experiments, the gonadotrope fraction showed 23 ± 3-fold more luciferase activity than unpurified dispersed cells. These data are consistent with gonadotropes containing all the activity for mut-o*FSHBLuc*. Therefore, specific activities in the pituitary column of Table 1 could be justifiably multiplied by 23-fold to represent only gonadotrope tissue because gonadotropes accounted for only 4.3% (1/23rd) of pituitary tissue on average. Using these new calculations, gonadotrope-specific expression was 644:1 (liver), 805:1 (gonads), 6440:1 (spleen), 2147:1 (lung), and 86:1 (brain) for male mice from the M248 transgenic line.

Expression of mut-oFSHBLuc did not increase at estrus

The data in Fig. 4 show that expression of wtLj-o*FSHBLuc* was increased at the time of estrus by 3- to 25-fold, with an average value of 10.4 ± 6.8. This widely variant value was similar to that found for 10 of the wild-type constructs previously reported (10.2 ± 2) (11, 12). The data in Fig. 4 show that no increase in expression occurred for mut-o*FSHBLuc* during estrus compared with diestrus. The mean ratio ± SEM for mut-o*FSHBLuc* was 0.7 ± 0.2, which indicates no increase in expression at estrus.

Activin did not induce mut-oFSHBLuc in primary pituitary cultures

The data in Fig. 5A (*WT Mouse FSH*) are similar to those in Fig. 1, except that mouse pituitary cultures were used instead of ovine pituitary cultures. Follistatin (250 ng/ml) decreased FSH secretion by an average of 71%, indicating that follistatin-sensitive factors, presumably activins, were stimulating the majority of endogenous FSH expression/secretion in mouse pituitary cultures. FSH was measured by RIA. Data in Fig. 5B (*WT-Luc*) show similar inhibition by follistatin of the wtLj-o*FSHBLuc* transgene, which is known

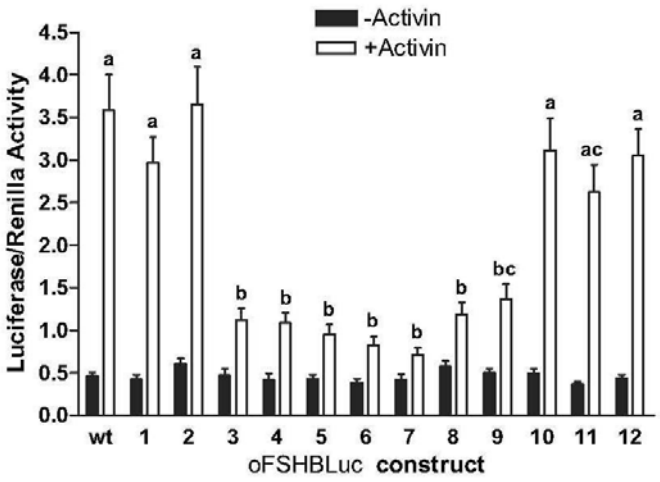

FIG. 2. Basal and activin-induced expression of o*FSHBLuc* constructs in LβT2 cells. Cells were transiently cotransfected with an internal standard (*Renilla*) plus wild-type (wt) o*FSHBLuc* or 12 different mutant o*FSHBLuc* constructs. Cultures were treated with or without 50 ng/ml activin A for 22 h, and assayed for luciferase and *Renilla* activities. The luciferase/*Renilla* ratios of activities are reported as the mean ± SEM of three independent experiments each assayed in triplicate. *Bars with different letters* are significantly different ($P < 0.05$). *Bars with the same letter* are not different from each other ($P > 0.05$). The wild-type sequence appears at the *top of the table*, and all 12 mutations (*bold letters*) appear *below* it. The putative Runx1 and SBE sites are labeled.

					Runx1		SBE						
wt	-175	ATC	TAC	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGG	CTT	-143
1		A**AG**	**ATC**	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGG	CTT	
2		A**AC**	**GCG**	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AGG	CTT	
3		ATC	TAC	T**CG**	**AG**T	TAG	ACT	ACT	TTA	GTA	AGG	CTT	
4		A**AG**	**ATC**	TGC	**TG**T	TAG	ACT	ACT	TTA	GTA	AGG	CTT	
5		A**AG**	**ATC**	TGC	GT**G**	**G**AG	ACT	ACT	TTA	GTA	AGG	CTT	
6		ATC	TAC	TGC	GTT	**AGA**	**T**CT	ACT	TTA	GTA	AGG	CTT	
7		ATC	TAC	TGC	GTT	**TCT**	A**GA**	ACT	TTA	GTA	AGG	CTT	
8		ATC	TAC	TGC	GTT	TAG	A**TA**	**T**CT	TTA	GTA	AGG	CTT	
9		ATC	TAC	TGC	GTT	TAG	AC**G**	**C**CT	TTA	GTA	AG**A**	**TC**T	
10		ATC	TAC	TGC	GTT	TAG	ACT	A**AG**	TTA	GTA	AG**A**	**TC**T	
11		ATC	TAC	TGC	GTT	TAG	ACT	ACT	TT**C**	**T**TA	AG**A**	**TC**T	
12		ATC	TAC	TGC	GTT	TAG	ACT	ACT	TTA	GTA	AG**A**	**TC**T	
bp #		-175 to -170		-169 to -160			-159 to -150				-149 to -143		

to be induced by activin and inhibited by follistatin. Data in Fig. 5, C and D, show relatively low expression of mut-o*FSHBLuc* in pituitary cultures from M231 or M248 transgenic mouse lines. Expression was not inhibited by follistatin, suggesting that they are not induced by activin.

Finally, the data in Fig. 5, C and D, show that neither activin A nor GnRH individually induced expression of mut-o*FSHBLuc* in primary pituitary cultures previously treated with follistatin for 16 h (*i.e.* deprived of activin-like paracrine stimulation). The combined effects of both activin and GnRH significantly increased expression of mut-o*FSHBLuc* by approximately 90% within the 6-h treatment period.

Discussion

Previous studies in rats showed that iv injections of either follistatin (80 μg) or inhibin (50 μg) decreased serum FSH by 60% (3). These studies showed that FSH production was significantly dependent on activins *in vivo* but were not designed to determine the full extent of this dependency. More recent studies focused on induction of FSH by activin and pulsatile GnRH with perifused rat pituitary cultures (Fig. 1 of Ref. 17) or activin alone with transformed LβT2 gonadotropes (Fig. 2 of Ref. 16). These studies indicate that expression of rodent FSH is heavily dependent on activin, perhaps 95–99% dependent, regardless of GnRH treatment.

Figure 1 of this study reinforces the aforementioned concept, especially for sheep because follistatin (225 or 675 ng/ml) suppressed FSH expression by 95 or 99%, respectively, in primary ewe pituitary cultures. It was considered important to show this level of inhibition for ovine FSH because it was the ovine *FSHB* gene studied here. It is not fully known what factors are produced in static cultures of mixed pituitary cells to stimulate expression of FSH, but evidence suggests that highly potent activin B (2) and less potent bone morphogenetic proteins (16) are made in gonadotropes to induce FSH in an autocrine manner. A recent report indicates that activin A and bone morphogenetic protein 2 can synergistically stimulate *FSHB* transcription (18). Stimulation from autocrine sources implies that high levels of follistatin would be necessary to rapidly intercept autocrine inducers just as they leave the plasma membrane to bind their cognate receptors. These high levels of follistatin can only be achieved *in vitro*. In this study high levels of follistatin were used to inactivate activins or other TGFβ family members, which blocked 99% FSH expression in primary ewe pituitary cultures.

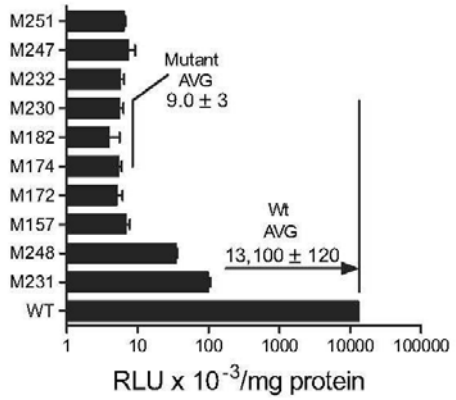

FIG. 3. Pituitary expression of mut-o*FSHBLuc* (see mutant 3 in Fig. 2) in 10 transgenic mouse lines. Expression of luciferase was measured in male pituitaries and is reported as the mean ± SEM of five to 10 mice taken from different litters (data for seven lines came from nine mice each). Pituitary tissue was harvested from mice between 8 and 20 wk old. The most active mut-o*FSHBLuc* lines (M231 and M248) were selected for further study (see Figs. 4 and 5). AVG, Average; WT, wild type.

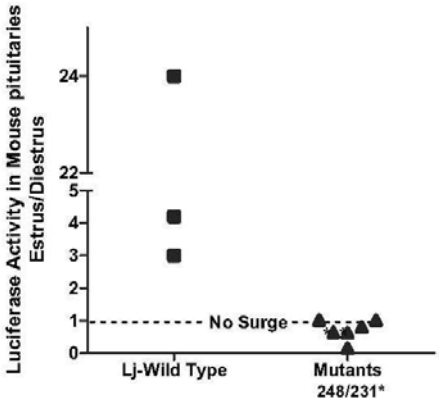

FIG. 4. Expression of mut-o*FSHBLuc*-ΔRunx1 did not increase at estrus as did wild-type transgenes. Each *triangle* shown comprised results from six to eight littermates. A total of 46 mut-o*FSHBLuc*-ΔRunx1 mice were used (M231 = 30 mice; M248 = 16 mice), and 24 wtLj-o*FSHBLuc* mice were used with triplicate values for both luciferase and protein in all cases. Because diestrus values varied significantly between litters, results were always normalized by reporting the ratio of estrus-diestrus for each wild-type or mutant litter. Student's *t* test was used to show that there was a significant difference between the wild-type and mutant responses ($P < 0.01$).

It could be argued that such supraphysiological levels of follistatin inhibit FSH expression through a nonspecific toxic mechanism. However, there are no reports showing that follistatin at 250 ng/ml is toxic to gonadotropes, and this concentration has been used routinely to block FSH expression in LβT2 cells (19). Furthermore, follistatin produced a normal saturation-type inhibition curve (IC_{50} = ~20 ng/ml; Fig. 1). The dose-response curve for follistatin-mediated inhibition that was constructed from Fig. 1 (data not shown) contained no discontinuities to suggest the presence of nonspecific toxic effects. In addition, we showed that the effects of all concentrations of follistatin were fully reversed within 24 h after follistatin withdrawal. Nevertheless, all levels of follistatin used in this study were supraphysiological, and it is possible that some effects were due to rapidly reversible toxicity.

The data in Fig. 3 show that changing four nucleotides in 4741 bp of the ovine *FSHB* promoter decreased luciferase expression *in vivo* to an average of 9×10^3 RLU/mg protein from $13{,}100 \times 10^3$ RLU/mg protein for wtLj-o*FSHBLuc*. In addition, low expression of the mutant transgene should be compared with previously reported data from 10 wild-type transgenes ($16{,}100 \times 10^3$ RLU/mg protein) (11, 12). Therefore, this small mutation caused a 99.93% reduction in luciferase expression. Because this mutation caused no change in basal expression of mut-o*FSHBLuc* in LβT2 cells (Fig. 2), there was no evidence to link any of this decrease to a change in basal expression, which suggested that the entire decrease was due to a complete and absolute withdrawal of activin like stimulation. These data are consistent with the results shown in Fig. 1, in which follistatin inhibited culture-mediated induction of FSH by 95–99%.

The almost absolute blockade of FSHB expression caused by mutating the −169 to −165-bp site seems so large, however, that it raises a question about this site being involved in basal expression as well as activin induction. It could even be possible that the mutation itself created an inhibitory site on the *FSHB* promoter that artificially decreased expression. However, the Consite computer program (7) did not associate any protein (activator or inhibitor) with the mutated site. Furthermore, Runt or forkhead family members have never been associated with basal expression before. Nevertheless, it is possible that this particular site participates in both basal and activin-stimulated *FSHB* expression. This could explain the inhibition caused by Smad3 small interfering RNAs at both basal and activin-induced levels at the putative SBE site (20). An analysis of the proteins that bind this sequence and the putative SBE site juxtaposed to it will be needed to reveal the full nature and importance of these sites.

Mutating sequences from −169 to −165 bp (the putative Runx1 binding site) prevented mut-o*FSHBLuc* from being induced during the estrous surge. This induction during estrus was observed for wtLj-o*FSHBLuc* and all other wild-type transgenes studied to date in our laboratory (11, 12). For example, the increase in luciferase activity at estrus for wtLj-o*FSHBLuc* was 10 ± 7-fold (range 3–24), which was similar to that previously reported for seven similar wild-type transgenic lines (10 ± 2-fold increase with a range from 3–19). This increase mimicked that of endogenous mouse FSH (21–23), which is associated with the recruitment of follicles for development during subsequent reproductive cycles (24, 25). Studies in rats have concluded that activin B drives this FSH surge at estrus (26–28). If mutating the putative *Runx1* site destroyed basal expression of FSHB, the data in Fig. 4 simply reflect the fact that induction cannot occur in a gene that is transcriptionally inactive. However, if basal expression remained unchanged, the data presented here support the idea that activin, working through the −169 to −165 bp site, helps create the secondary FSH surge.

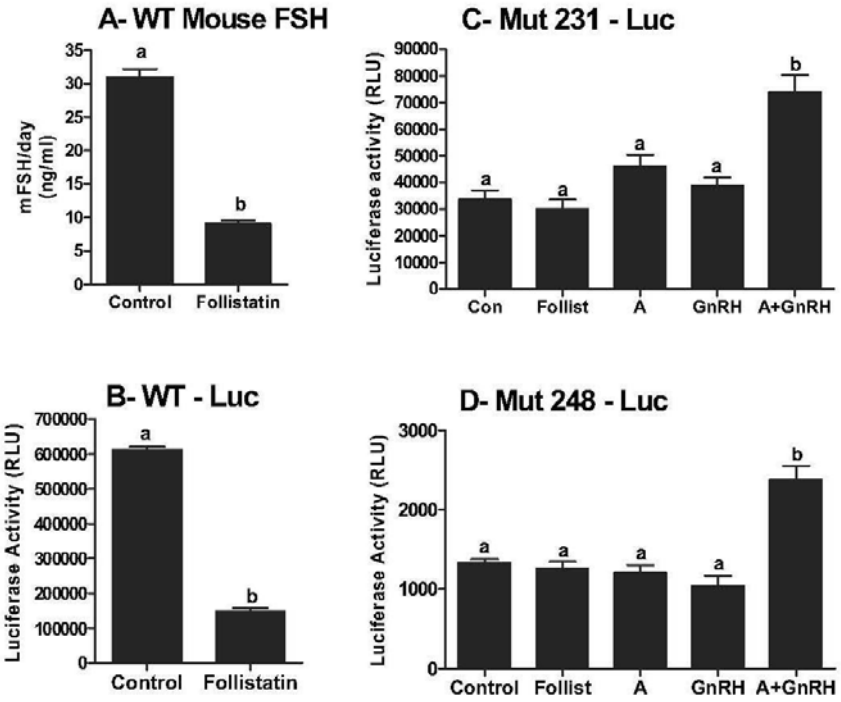

FIG. 5. Regulation of endogenous FSH, wtLj-o*FSHBLuc*, or mut-o*FSHBLuc* in mouse pituitary cultures by follistatin (Follist), activin, and/or GnRH. A, Mouse pituitary cultures were treated with 250 ng/ml follistatin on d 2 (0900 h), and culture media were collected on d 3 (0900 h) and assayed for FSH by RIA. Follistatin decreased FSH by 71 ± 3%. B, Pituitary cultures were prepared from mice carrying the wtLj-o*FSHBLuc* transgene, cultured 2 d, and then treated with or without follistatin for 24 h before being assayed for luciferase (Luc) activity. C, Pituitaries from mice carrying the mut-o*FSHBLuc* transgene (M231 line) were cultured 1.5 d and treated without (control) or with 250 ng/ml follistatin for 16 h to deprive them of activin stimulation from autocrine/paracrine factors made in pituitary culture. They were then treated with follistatin again (follist), activin (50 ng/ml), GnRH (1 nM), or activin plus GnRH for another 6 h before being assayed for luciferase activity. D, Same as panel C except pituitaries from M248 transgenic mice were used. The results represent the mean ± SEM of three replicate experiments each assayed in triplicate. Means that are significantly different from each other are labeled with *different letters* (a or b) where $P < 0.05$. Con, Control.

The putative Runx1 binding site was discovered experimentally while studying deletion and point mutants of ovine *FSHBLuc* constructs in LβT2 cells (Fig. 2). Computer analysis of transcription binding sites, an imprecise science still in its infancy, identified the region from −171 to −163 bp as a likely Runx1 binding site (81%; score = 6.0) (7). This was useful information because Runx1 is known to interact specifically and strongly with Smad3 to promote the proper growth and differentiation of hematopoietic cells (8). This linkage suggested that ovine FSHB might be induced by activin working through a similar complex containing a Runt family member plus Smad3. Furthermore, the putative Runx1 binding site is highly conserved for sheep, human, pig, buffalo, and cow (29). There is one nucleotide change in the pig *FSHB* promoter, but this natural mutation creates an even better Runx1 binding site according to the Consite analysis.

Interestingly, the single mutation in the pig *FSHB* promoter also creates a putative binding site for FREAC-4, a forkhead protein that may also interact with Smads, although this has not been proven for this particular forkhead protein at this specific site. Another interesting finding is that mutating GC to TA at positions −168 and −167 bp created a putative forkhead-1 binding site. This construct significantly increased responsiveness to activin (48-fold induction), while barely altering basal expression in LβT2 cells (Su, P., unpublished data). In addition, a single mutation from T to C at position −164 bp created a palindromic Smad binding site that permitted activin to induce this construct 38-fold in LβT2 cells, but basal expression was increased approximately 20-fold (Su, P., unpublished data). Therefore, depending on the nature of DNA sequences and protein factors surrounding the putative Runx1/SBE binding sites, this promoter region might accommodate several forkhead-related proteins known to interact with Smads and might even act as a palindromic SBE element like the one found in rodent promoters for *FSHB* at −266 bp (29). As noted previously, further analysis of the proteins that bind this region will be required to determine the true nature of transcription factor interactions that help drive activin-mediated induction, and possibly basal expression, of the ovine *FSHB* gene.

It might appear that identifying the transcription factors that bind to sequences between −171 and −158 bp on the ovine *FSHB* promoter would be relatively easy using EMSAs, but this perception would be incorrect. No laboratory has yet demonstrated activin-induced binding of any protein to either the putative SBE or its adjacent site identified here. The entire 24-h time course of activin induction has been analyzed without success (data from our laboratory). There is one report of a protein-DNA complex with this promoter region, but this interaction was not induced by activin (5). Nevertheless, the *in vitro* and *in vivo* data in this report about the putative Runx1 binding site plus data from others (SBE site) indicate that the ovine FSHB promoter region from −171 to −158 bp is critical for *FSHB* expression in sheep and presumably in humans and other mammals that contain this highly conserved promoter sequence.

Cell-specific expression is important for every gene. It is possible in many cases for proximal enhancers to play a significant role in cell-specific expression and that appears to

be partly true for the putative Runx1 site. Pituitary specific expression for mut-o*FSHBLuc* appears to be 66% less than for wt-o*FSHBLuc* (11) or wtLj-o*FSHBLuc*. Expression of wt-o*FSHBLuc* (11) or wtLj-o*FSHBLuc* (unpublished data from our laboratory) is less than 1% pituitary expression in the liver, gonads, spleen, or lung, but expression of mut-o*FSHBLuc* was approximately 3% pituitary expression in the liver and gonads; however, expression in the spleen and lung were still ≤1% compared with pituitary expression. For the liver and gonads this represents a 66% decline in gonadotrope-specific expression suggesting that the putative Runx1 site has some effect on cell-specific expression. Nevertheless, data from purified gonadotropes expressing mut-o*FSHBLuc* show that gonadotrope-specific expression is still favored 644:1 (see *Results*) when comparing gonadotrope expression to expression in either the liver or gonads. Pituitary specific expression is even higher when considering the spleen (6440:1) or lung (2147:1). Expression of ovine *FSHBLuc* constructs in brain tissue is another matter entirely. The original wild-type construct (wt-o*FSHBLuc*) was often expressed in brain tissue at high levels (pituitary-brain = 100:1, 100:30 100:270) (11). The mutant transgene, mut-o*FSHBLuc*, was also expressed at significant levels in the brain (pituitary-brain = 100:8 or 100:22). In this regard, expression of mut-o*FSHBLuc* appeared even more specific compared with the original wild-type transgenes with regard to brain tissue. These data comparing pituitary and brain expression are difficult to comprehend and have not been helpful in understanding cell-specific expression of the ovine *FSHB* gene. The overall results indicate that the putative Runx1 site has some influence over gonadotrope-specific expression, at least in the liver and gonads, but it seems to play only a minor role overall.

Finally, GnRH and activin often interact at a molecular level to alter *FSHB* expression, but these interactions are not well understood. Our laboratory previously reported that isolating primary gonadotropes from activin for 17 h (follistatin treatment) permits subsequent treatment with activin and GnRH to synergistically induce wt-o*FSHBLuc* during a 4-h period (12). Moreover, recent studies with the mouse *FSHB* promoter show that GnRH and activin can cooperate synergistically when critical AP-1 and Smad binding sites are multimerized (30).

The data in Fig. 5 show that GnRH and activin can synergistically increase expression of mut-o*FSHBLuc*, but this occurred in a construct that lacked the putative Runx1 site and was, presumably, incapable of responding to activin directly (Figs. 2–4). These data suggest that GnRH and activin cooperated either through the one remaining Smad binding site or in a general way that did not involve specific interactions on the ovine *FSHB* promoter. Recent evidence indicates that activin dramatically alters the responsiveness of LβT2 gonadotropes to GnRH in a global sense (31), so it is possible that global effects of GnRH and activin caused the synergistic induction of mut-o*FSHBLuc* shown in Fig. 5. These data are not meant to imply that activin and GnRH do not cooperate with each other through signaling pathways that complement each other directly at the ovine *FSHB* promoter, but it does indicate there are ways for activin and GnRH to cooperate through global actions in gonadotropes.

In summary, this study presents data that are consistent with the concept that 99.9% of ovine *FSHB* expression *in vivo* depends on sequences between −171 and −165 bp on the promoter of ovine *FSHB* promoter/reporter gene mutants in LβT2 gonadotropes. It is shown here that this element was essential *in vivo* and was required for the estrous surge of FSH in mice. It seems to have had little effect on pituitary specific expression. Finally, it was shown that activin and GnRH cooperatively increased *FSHB* expression, even in the absence of a functional activin response element on the ovine *FSHB* promoter.

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References

1. Kumar TR, Wang Y, Lu N, Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204
2. Corrigan AZ, Bilezikjian LM, Carroll RS, Bald LN, Schmelzer CH, Fendly BM, Mason AJ, Chin WW, Schwall RH, Vale W 1991 Evidence for an autocrine role of activin B within anterior pituitary cultures. Endocrinology 128: 1682–1684
3. DePaolo LV, Shimonaka M, Schwall RH, Ling N 1991 *In vivo* comparison of the follicle-stimulating hormone-suppressing activity of follistatin and inhibin in ovariectomized rats. Endocrinology 128:668–674
4. Matzuk MM, Kumar TR, Bradley A 1995 Different phenotypes for mice deficient in either activins or activin receptor type II. Nature 374:356–360
5. Bailey JS, Rave-Harel N, McGillivray SM, Coss D, Mellon PL 2004 Activin regulation of the follicle-stimulating hormone β-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol Endocrinol 18: 1158–1170
6. Su P, Miller WL 2004 Identification of an activin response element on the promoter of ovine follicle stimulating hormone β-subunit. Proc Society for the Study of Reproduction, New Orleans, LA, 2004 (Abstract 371)
7. Lenhard B, Sandelin A, Mendoza L, Engstrom P, Jareborg N, Wasserman WW 2003 Identification of conserved regulatory elements by comparative genome analysis. J Biol 2:13
8. Jakubowiak A, Pouponnot C, Berguido F, Frank R, Mao S, Massague J, Nimer SD 2000 Inhibition of the transforming growth factor β1 signaling pathway by the AML1/ETO leukemia-associated fusion protein. J Biol Chem 275:40282–40287
9. Ji C, Eickelberg O, McCarthy TL, Centrella M 2001 Control and counter-control of TGF-β activity through FAST and Runx (CBFa) transcriptional elements in osteoblasts. Endocrinology 142:3873–3879
10. Attisano L, Silvestri C, Izzi L, Labbe E 2001 The transcriptional role of Smads and FAST (FoxH1) in TGFβ and activin signaling. Mol Cell Endocrinol 180:3–11
11. Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL 2001 The promoter for the ovine follicle-stimulating hormone-β gene (FSHβ) confers FSHβ-like expression on luciferase in transgenic mice: regulatory studies *in vivo* and *in vitro*. Endocrinology 142:2260–2266
12. Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL 2001 Transcriptional regulation of the ovine follicle-stimulating hormone-β gene by activin and gonadotropin-releasing hormone (GnRH): involvement of two proximal activator protein-1 sites for GnRH stimulation. Endocrinology 142:2267–2274
13. Beggs MJ, Miller WL 1989 GnRH-Stimulated LH release from ovine gonadotrophs in culture is separate from phorbol ester-stimulated LH release. Endocrinology 124:667–674

14. Wu JC, Su P, Safwat NW, Sebastian J, Miller WL 2004 Rapid, efficient isolation of murine gonadotropes and their use in revealing control of follicle-stimulating hormone by paracrine pituitary factors. Endocrinology 145:5832–5839
15. Miller WL, Knight MM, Grimek HJ, Gorski J 1977 Estrogen regulation of follicle stimulating hormone in cell cultures of sheep pituitaries. Endocrinology 100:1306–1316
16. Huang HJ, Wu JC, Su P, Zhirnov O, Miller WL 2001 A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology 142:2275–2283
17. Weiss J, Crowley Jr WF, Halvorson LM, Jameson JL 1993 Perifusion of rat pituitary cells with gonadotropin-releasing hormone, activin, and inhibin reveals distinct effects on gonadotropin gene expression and secretion. Endocrinology 132:2307–2311
18. Lee KB, Khivansara V, Santos MM, Lamba P, Yuen T, Sealfon SC, Bernard DJ 2007 Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone β subunit transcription. J Mol Endocrinol 38:315–330
19. Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL 2001 Cell-specific regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell model. Endocrinology 143:3243–3249
20. Suszko MI, Balkin DM, Chen Y, Woodruff TK 2005 Smad3 mediates activin-induced transcription of follicle-stimulating hormone β-subunit gene. Mol Endocrinol 19:1849–1858
21. Ortolano GA, Haisenleder DJ, Dalkin AC, Iliff-Sizemore SA, Landefeld TD, Maurer RA, Marshall JC 1988 Follicle-stimulating hormone β subunit messenger ribonucleic acid concentrations during the rat estrous cycle. Endocrinology 123:2946–2948
22. Hotchkiss J, Knobil E 1994 The menstrual cycle and its neuroendocrine control. In: Knobil E, Neill JD, Greenwald GS, Markert CL, Pfaff DW, eds. The physiology of reproduction. Vol 2. 2nd ed. New York: Raven Press; 712, 713, 726
23. Goodman RL 1994 Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neill JD, Greenwald GS, Markert CL, Pfaff DW, eds. The physiology of reproduction. Vol 2. 2nd ed. New York: Raven Press; 662, 664
24. Haughian JM, Ginther OJ, Kot K, Wiltbank MC 2004 Relationships between FSH patterns and follicular dynamics and the temporal associations among hormones in natural and GnRH-induced gonadotropin surges in heifers. Reproduction 127:23–33
25. Hoak DC, Schwartz NB 1980 Blockade of recruitment of ovarian follicles by suppression of the secondary surge of follicle-stimulating hormone with porcine follicular fluid. Proc Natl Acad Sci USA 77:4953–4956
26. DePaolo LV, Bald LN, Fendly BM 1992 Passive immunoneutralization with a monoclonal antibody reveals a role for endogenous activin-B in mediating FSH hypersecretion during estrus and following ovariectomy of hypophysectomized, pituitary-grafted rats. Endocrinology 130:1741–1743
27. Woodruff TK, Besecke LM, Groome N, Draper LB, Schwartz NB, Weiss J 1996 Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. Endocrinology 137:5463–5467
28. Besecke LM, Guender MJ, Sluss PA, Polak AG, Woodruff TK, Jameson JL, Bauer-Dantoin AC, Weiss J 1997 Pituitary follistatin regulates activin-mediated production of follicle-stimulating hormone during the rat estrous cycle. Endocrinology 138:2841–2848
29. Kumar TR, Schuff KG, Nusser KD, Low MJ 2006 Gonadotroph-specific expression of the human follicle stimulating hormone β gene in transgenic mice. Mol Cell Endocrinol 247:103–115
30. Coss D, Yaphockun KKJ, Mellon PL, Mechanisms of synergistic induction of FSHβ by GnRH and activin in LβT2 cells. Program of the 88th Annual Meeting of The Endocrine Society, Boston, MA, 2006 (Abstract P3-276)
31. Zhang H, Bailey JS, Coss D, Lin B, Tsutsumi R, Lawson MA, Mellon PL, Webster NJ 2006 Activin modulates the transcriptional response of LβT2 cells to gonadotropin-releasing hormone and alters cellular proliferation. Mol Endocrinol 20:2909–2930

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CHAPTER IV

Activin induction of the ovine follicle-stimulating hormone β-subunit is mediated by Smad4 and a forkhead box transcription factor

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ABSTRACT

Follicle stimulating hormone (FSH) is a α/β glycoprotein produced in pituitary gonadotropes of all vertebrates and is required for egg maturation, and optimal sperm performance. Activin potently induces FSH by inducing transcription of its rate-limiting β-subunit (*FSHB*). Four nucleotides of the o*FSHB* promoter (–168 bp to –165 bp) are necessary for 99.9 % of o*FSHB* expression *in vivo* and 70 % of its progressive induction by activin in LβT2 gonadotropes over a 24 hr period. These 4 nucleotides form part of a putative forkhead box (FOX) binding site juxtaposed upstream to a single-copy (4 bp) Smad binding element (SBE), both of which are associated with activin action. Smad4 from LβT2 cells did not bind the wild type o*FSHB* SBE in electrophoretic mobility shift assays, but did bind a palindromic SBE derived from the o*FSHB* SBE. Binding increased 2.6-fold over 20 h without or with activin (25 % additional increase with activin) and was competed 85 % with the native o*FSHB* sequence indicating Smad4 has high affinity for the native promoter. Additionally, a dominant negative inhibitor of Smad4 reduced activin induction of oFSHB in LBT2 cells by 62 %, indicating that Smad4 is important for activin induction. A second Smad, Smad3, bound transiently to the palindromic SBE (6-fold increase by activin at 2 h). Dominant negative inhibition of Smad3 (3SA) and depletion of Smad2 by siRNA did not alter activin induction of o*FSHB* suggesting Smads 2 and 3 may not be involved. A p38 inhibitor blocked induction of o*FSHB* after 8 h, dividing the 24 hr induction by activin into two phases. This suggested that an activin-regulated early gene product is required for the second phase of oFSHB induction. It was found that one forkhead gene, *FOXQ1*, was increased 4.5-fold 8 h after activin treatment which correlated nicely with the second phase

of oFSHB induction uncovered by the p38 inhibitor. FOXQ1 is only one of 43 FOX family members, however, so further studies are required to prove that FOXQ1 is a key driver of FSH production and that it partners with Smad4 to induce o*FSHB* transcription.

INTRODUCTION

Follicle Stimulating Hormone (FSH) is essential for folliculogenesis in females and enhances the performance and maturation of sperm in males (1). It is an α/β heterodimer that is produced only within pituitary gonadotrope cells. Production and secretion of FSH depends upon transcription of its unique β-subunit, *FSHB*, which is carefully regulated by more than six reproductive hormones. Increased *FSHB* expression is controlled primarily by members of the transforming growth factor-β (TGFβ) superfamily (2-5) and GnRH (6), whereas inhibin (6, 7) and follistatin (6, 8) decrease expression. Activin, a member of the TGFβ superfamily, is a major inducer of *FSHB* transcription that is required for at least half of FSH production *in vivo* (9, 10). Many studies are thus focused on defining the molecular mechanisms used by activin to transcriptionally regulate *FSHB*, which is rate-limiting for overall FSH production.

Activin exists as three possible isoforms that are homo- or heterodimers of activin β_A- and β_B-subunits (11), however, activin A (homodimer of β_A-subunits) is the most potent inducer of *FSHB* transcription (12). It exerts its effects by first binding to activin type II serine/threonine kinase receptors (ActRII or ActRIIB), which recruit and phosphorylate activin type I receptors, such as ALK4 (activin receptor-like kinase 4). The type I receptor then propagates the signal downstream, ultimately enhancing *FSHB* expression. Smad2 and 3 are widely recognized as activin- and TGFβ-activated transcription factors. Following receptor-mediated activation Smad2 and/or 3 form a complex with the common-mediator (Co-Smad) Smad4, which translocates to the nucleus and regulates gene expression. Gene targeting occurs through Smad binding to specific DNA sequences known as Smad binding

elements (SBEs) that exist as 8 bp palindromes (5'-GTCTAGAC) or 4 bp single-copy sites (5'-GTCT or 5'-AGAC) (13, 14). Binding to these sites, however, is rather weak and non-specific. Therefore, additional transcription factors are often required for high affinity binding, and cell- and promoter-specific gene regulation. Indeed, Smads 2, 3 and 4 are known to interact with numerous DNA-binding transcription factors, recently reviewed in detail by Ross and Hill (15).

Activin-induced expression of both mouse and rat *FSHB* is undoubtedly dependent on Smads 2, 3 and 4 (16-20), presumably because their promoters harbor a naturally occurring palindromic SBE. However, our laboratory, through a collaborative effort, provided significant evidence to suggest the importance of additional, non-Smad transcription factors in activin regulation of mouse *FSHB* (m*FSHB*). Using a recently developed method to purify gonadotropes from transgenic mouse pituitaries (21), we surprisingly observed that unlike potent induction by activin, TGFβ inhibits m*FSHB* expression by 95 % (see Chapter I, Fig. 7A, [22]). Because both activin and TGFβ activate Smads 2 and 3, this novel finding demonstrated that *FSHB* induction by activin likely depends on alternate transcription factors. If it were merely driven by Smads binding to a constitutively expressed partner protein, then similar effects of activin and TGFβ would have occurred.

Unlike rodent species, the ovine *FSHB* (o*FSHB*) promoter does not contain a palindromic SBE, and the importance of Smads in o*FSHB* induction by activin is not well defined. Activation of Smad3 does not seem important because overexpression of an unphosphorylatable form of Smad3 cannot abrogate activin induction of an o*FSHB* promoter/reporter construct (o*FSHBLuc*) in LβT2 cells (see Chapter II, [23]). The

importance of Smads cannot be eliminated, however, primarily because two single-copy SBEs are critical for maximum induction of o*FSHB* by activin (24, 25). Although which Smads bind to these sites remains questionable. Therefore, the role(s) of Smad2 and/or 4 in mediating activin induction of o*FSHB* deserves investigation.

There is also evidence, generated by our laboratory, for the involvement of a transcription factor other than Smads in mediating activin induction of o*FSHB*. The first indication that other transcription factors are critical for activin action was due to different kinetics of induction between o*FSHBLuc* and a Smad-dependent reporter, p3TPLuc, in LβT2 cells. Induction of p3TPLuc occurs rapidly and transiently (2-8 hrs), consistent with the level of Smad2/3 activation in LβT2 cells (16), whereas induction of o*FSHBLuc* occurs slowly and progressively, with maximal induction at 22 hrs (see Chapter II, Fig. 3, [23]). These differences indicate that Smads alone are not sufficient for triggering activin induction of o*FSHBLuc*. They also suggested that o*FSHB* synthesis likely depends on an activin-regulated early response gene(s) whose product(s) could account for the slow and maximal level of induction.

The second indication was through the identification of a novel site, spanning -171 bp to -163 bp of the o*FSHB* promoter. This site is juxtaposed directly upstream to a single-copy SBE at -162 bp that, although important for activin induction of o*FSHBLuc*, has not been shown directly to bind Smads. When mutated, activin induction of o*FSHBLuc* in LβT2 cells is reduced by almost 70 %, and in transgenic mice, the mutation decreases o*FSHBLuc* expression by 99.9 % (see Chapter III, Figs. 2 and 3, [25]). These studies indicate that physiologically, these sequences and the proteins that bind them are critical for activin

action. This site was identified as a putative Runx1 or forkhead-box (FOX) binding site because both bind similar sequences. Importantly, members of both Runx and FOX protein families are reported to interact with Smads (26-28). Therefore, either Runx or FOX proteins may act through this site in association with Smads to induce o*FSHB* in response to activin. The identity of this factor is currently unknown.

In addition to the potential role of Smads and Smad-associated transcription factors, the mitogen-activated protein kinase (MAPK) pathway is also necessary for activin-induced o*FSHB* expression. Activin induction of o*FSHB* relies heavily on the MAPKKK, TGFβ-activated kinase 1 (TAK1,) and its downstream MAPK target, p38 (see Chapter II, Figs. 5A and 8C, [23]). Although important, the MAPK pathway alone also does not explain the slow induction of o*FSHBLuc* by activin because, like Smad-only responses, direct MAPK-mediated gene activation is typically rapid (29). Many targets are immediate early genes (IEGs) that can be transcription factors important for mediating transcription of late-responding genes, such as o*FSHB*. Because *Runx* genes are generally regarded as constitutively expressed, an activin-regulated member of the *FOX* family may be important for driving the late and maximal induction of o*FSHB* by activin. The studies presented here are focused first on clarifying the role(s) of Smads and p38 in o*FSHB* regulation by activin, and importantly, on identifying a FOX member that may be central to controlling FSH production.

MATERIALS AND METHODS

I. Reagents

Recombinant human activin A was obtained from R&D Systems, Inc. (Minneapolis, MN). Dulbecco's modified Eagle medium (DMEM) with high glucose, L-glutamine and sodium pyruvate was purchased from Invitrogen Co. (Carlsbad, CA). Fetal bovine serum was from Hyclone Laboratories, Inc. (Logan, UT). Dithiothreitol (DTT) and phenylmethylsulphonylfluoride (PMSF) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Anti-Smad3 (FL-425x) and anti-Smad4 (B-8x) antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). FuGENE 6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). The p38 inhibitor, SB203580 was purchased from Calbiochem (San Diego, CA). The Luciferase Assay System and 5x Passive Lysis Buffer were purchased from Promega (Madison, WI). TRI Reagent was from Molecular Reseach Center Inc. (Cincinnati, OH). The iScript cDNA synthesis kit was from Bio-Rad Laboratories, Inc. (Hercules, CA), and Taqman Universal PCR Master Mix was purchased from Applied Biosystems (Foster City, CA).

II. Reporter plasmids and expression vectors

The ovine reporter plasmid, o*FSHBLuc*, has been described previously (30). The mouse reporter plasmid, m*FSHBLuc*, the Smad2 RNAi construct and its empty vector, pBS/U6, have been previously described (16) and were a generous gift from Dr. Daniel J. Bernard (McGill University, Montreal, Quebec, Canada). The C-terminally truncated dominant negative (DN) Smad4 (Smad4 1-514) construct, driven by the cytomegalovirus

(CMV) promoter and its empty vector, pCMV, were kindly provided by Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY) (31).

III. Culture and transient transfection of LβT2 gonadotropes

LβT2 cells were provided by Dr. Pamela L. Mellon (University of California, San Diego, CA) and were cultured as described previously (23). For Fig. 2A and B, LβT2 cells were plated at a density of 25,000 cells per well in 96-well Primaria culture plates (Becton Dickinson, Franklin Lakes, NJ). Cells were transfected 24 hrs later in triplicate with 50 ng wild-type o*FSHBLuc* and were cotransfected with 50 ng of pCMV or DN-Smad4 (Fig. 2A), or 50 ng or pBS/U6 of Smad2 RNAi (Fig. 2B). In Fig. 2C, cells were transfected with 50 ng wild-type m*FSHBLuc*, and cotransfected with 50 ng of pBS/U6 or Smad2 RNAi. All transfections were performed with FuGENE 6 (Roche Applied Science) using 300 ng/well. Plasmids and FuGENE 6 were incubated in serum-free DMEM for 15 min at RT, then 50 µl was added to each well. After 24 hrs, cells were treated with or without 50 ng/ml activin A for 22 hrs and then assayed for luciferase activity.

For the p38 studies (Fig. 3), cells were transfected with 50 ng wild-type o*FSHBLuc* with FuGENE 6 using 150 ng/well. After 24 hrs, cells were treated for 1 hr with fresh media containing 50 µM SB203580 or an equivalent volume of DMSO as a control. SB203580 was prepared in DMSO at a stock concentration of 50 mM, so that each well contained 0.1 % DMSO. Cultures were then treated with additional media containing 50 µM SB203580 or DMSO, both with or without 50 ng/ml activin A. Cells were harvested every 2 hrs for 24 hrs, then assayed for luciferase activity.

IV. Luciferase assay

LβT2 cells in 96-well plates were lysed in 30 µl 1x Passive Lysis Buffer (Promega), and 10 µl was assayed for luciferase activity using an automated 1420 Victor-Light micro plate luminometer (Perkin-Elmer, Waltham, MA).

V. Isolation of nuclear extracts

LβT2 cells were plated in 6 ml DMEM at a density of 10 x 10^6 cells per plate in 100 mm Primaria culture dishes (Becton Dickinson). After 48 hrs, cells were treated with fresh media, with or without 50 ng/ml activin A for 1, 4 or 20 hrs, and nuclear extracts were isolated as previously described (32) with minor alterations to the protocol. Briefly, cells were washed once with 5 ml then scraped with 1.5 ml of ice-cold PBS, and transferred to a microfuge tube. Cells were pelleted at 5000 x g for 30 sec at RT and resuspended in 800 µl of cold Buffer A (10 mM HEPES-KOH, pH 7.9 at 4 C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF). After incubation on ice for 10 min, 25 µl of 10 % NP-40 was added, samples were vortexed for 10 sec, and centrifuged at 12000 x g for 10 sec at RT. Pellets were resuspended in 150 µl of cold Buffer C (20 mM HEPES-KOH, pH 7.9 at 4 C, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min. Cellular debris was removed by centrifugation at 12000 x g for 5 min at 4 C, and the supernatant fractions were saved and stored at -80 C. Protein concentrations were determined using the Qubit Fluorometer and Quant-it Protein Assay Kit (Invitrogen Co., Carlsbad, CA).

VI. Electrophoretic mobility shift assays (EMSA)

A double-stranded oligo corresponding to -171/-156 bp of the o*FSHB* promoter, with a T to C mutation at -164 bp (T164C, Table 1), was 5' end-labeled with ^{32}P-γATP (Perkin-Elmer) using T4 Polynucleotide Kinase (Epicentre Biotechnologies, Madison, WI). Nuclear proteins (5 μg) were incubated with 3.5 ng (40,000 cpm) of ^{32}P-labeled T164C in 25 mM HEPES (pH7.2), 1 mM KCl, 5 mM dithiothreitol, 10% glycerol and 500 ng salmon sperm DNA at RT for 20 min. In supershift experiments, reactions were assembled at RT and incubated for 10 min with 1 μg of Smad3 or Smad4 antibodies prior to the addition of probe. For Smad4 competition experiments (Fig. 1B), reactions were assembled at RT, incubated for 10 min with 100-fold excess of unlabeled T164C, palindromic SBE (SBE), wild-type o*FSHB* (Wt) or non-specific (NS) (Table 1) oligos, followed by an additional 10 min incubation with the Smad4 antibody prior to the addition of probe. The T164C, Wt and NS oligos were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and the SBE oligo (sc-2603) was purchased from Santa Cruz Biotechnology, Inc. Reactions were then run

Table 1: Oligonucleotides used in EMSA. Listed are the sense strand sequences of 16 bp double-stranded oligos used in EMSA. Only T164C, which corresponds to -171 bp to -156 bp of the o*FSHB* promoter and has a T to C mutation at -164 bp (shown in bold lowercase) was ^{32}P-labeled. All others were used as cold competitors (Fig. 1B). The SBE oligo contains a consensus palindromic Smad binding element (underlined). The Wt oligo corresponds to -171/-156 bp of the wild-type o*FSHB* promoter, and the NS oligo consists of random base pairs that are not homologous to the wild-type o*FSHB* promoter or a SBE.

Name	Sequence
T164C	5' - ACTGCGT**c**TAGACTAC
SBE	5' - AGTATGTCTAGACTGA
Wt	5' - ACTGCGTTTAGACTAC
NS	5' - CTAATACGACTCACTA

on 5 % native polyacrylamide gels (29:1 acrylamide:bis-acrylamide) in 40 mM Tris–HCl/195 mM glycine (pH 8.5) at 180 volts for 3 hrs at 4 C. Gels were dried for 1 hr using a Model 583 gel dryer (Bio-Rad Laboratories, Inc.) and scanned with a Molecular Dynamics Model 445 SI PhosphorImager (GE Healthcare, Waukesha, WI). Gels were visualized and bands quantatitated using Image Quant 5.0 (GE Healthcare).

VII. Quantifying *FOXQ1* mRNA levels

LβT2 cells were plated at a density of 25,000 cells per well in 96-well Primaria culture plates (Becton Dickinson). After 48 hrs, cells were treated with fresh media containing 50 ng/ml activin A. Following 0, 2, 3, 4, 6, 8, 12, 16 and 24 hrs of activin treatment, cells were harvested in 800 µl TRI Reagent (Molecular Research Center, Inc.) and total RNA was isolated according to the manufacturer's recommendations. RNA was converted to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.). Oligonucleotides for Taqman real-time PCR were designed for mouse cDNA for FOXQ1 and 18s ribosomal RNA (Table 2), which served as the internal control, using software from Integrated DNA Technologies, Inc. All Taqman probes were 5'-labeled with FAM and real-time PCR of all cDNA samples was performed at the same time. Real-time PCR was performed in duplicate on triplicate cDNA samples for each time point using an ABI 7300 Real-time PCR Cycler (Applied Biosystems). Reactions consisted of 1 µl of cDNA and 29 µl of 1x master mix, which consisted of 5 pmol each of primers and probes and 15 µl of 2x Taqman Universal PCR Master Mix (Applied Biosystems). Samples were incubated at 50 C for 2 min, 95 C for 10 min, and then for 40 complete cycles (95 C for 15 sec, and 60 C for 1

min). Threshold cycle (C_T) values were determined with Applied Biosystems software and used for relative quantitation with the $2^{-\Delta\Delta Ct}$ method (33).

Table 2: Real-time RT-PCR primers and probes. Listed are the sequences for the forward and reverse primers, and 5' FAM-labeled probes for mouse cDNA for FOXQ1 and 18s rRNA.

Primer/Probe Set	**Sequence**
FOXQ1	Forward: 5' - AGGACAGCACTGCACCAACTTCTA Reverse: 5' - AAAGGTTCTCCTCCGCTGGGATTT Probe: 5' - ACTCCACTCCTCACTTCACCCAACAA
18s rRNA	Forward: 5' - GAAACTGCGAATGGCTCATTAA Reverse: 5' - GAATCACCACAGTTATCCAAGTAGGA Probe: 5' - ATGGTTCCTTTGGTCGCTCGCTCC

RESULTS

I. Smad4 binds the wild-type o*FSHB* promoter

The sequences between -171 bp and -159 bp of the o*FSHB* promoter must bind activin-responsive proteins because this region is necessary for activin induction of *oFSHBLuc*. Initial attempts that used electrophoretic mobility shift assays (EMSA) to visualize binding of these proteins to a radiolabeled wild-type o*FSHB* double-stranded DNA oligo (Wt, Table 1), however, were unfruitful. Interestingly, making a T to C substitution at -164 bp created a consensus 8 bp palindromic SBE from -166 bp to -159 bp of the o*FSHB* promoter (see T164C, Table 1) that is known to bind Smads 3 and 4 in EMSAs (13). This mutation provided a reproducible method to investigate interactions between Smads 3 and 4, and this palindromic SBE in control or activin treated LβT2 nuclear extracts.

Depicted in Fig. 1A is a representative EMSA that shows the kinetics and composition of complexes formed between LβT2 nuclear extracts and the T164C probe under control and activin-stimulated conditions. Incubation of ^{32}P-labeled T164C with control treated LβT2 nuclear extracts resulted in little to no complex formation (Fig. 1A, lanes 2-4). A single, but faint complex appeared at 20 hrs (lane 4). After only 1 hr, activin caused a robust 7-fold increase in complex formation relative to control (compare lanes 2 and 5), which was transient because it fell to 3.5- and 2-fold at 4 and 20 hrs, respectively (lane 6 vs. 3, and 7 vs. 4). Although, some of this reduction can be attributed to the slight increase in binding under basal conditions (compare 1 hr and 20 hrs, lanes 2 and 4, respectively), the activin-stimulated binding at 20 hrs is clearly less than at 1 hr (lane 7 vs. 5).

Specific antibodies were used to determine if these complexes contained Smad3 and/or 4, but Smad2 was not investigated because it cannot directly bind DNA, nor bind to a palindromic SBE in EMSAs (13). Inclusion of a Smad3 antibody caused a supershift that was observed only after activin stimulation (*, lanes 11-13). Activin-induced Smad3 binding was also transient. Relative to control, Smad3 binding increased 6-fold after 1 hr of activin stimulation (lane 11), and based on an average of two EMSAs, the levels of Smad3 bound decreased by more than 50 % after 20 hrs (lane 13 vs. 11).

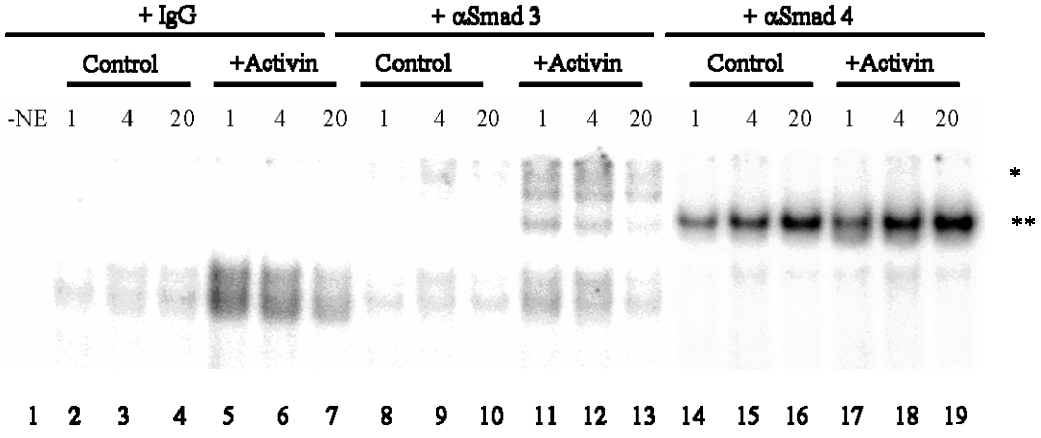


Figure 1A: Kinetics and composition of complexes formed between LβT2 nuclear extracts and T164C. EMSA was performed using no nuclear extracts (-NE; lane 1) or LβT2 nuclear extracts that had been treated with control media (Control) or activin (+Activin) for a period of 1, 4 or 20 hrs, and ^{32}P-labeled T164C (Table 1). Antibodies to Smad3 (+αSmad3; lanes 8-13) and Smad4 (+αSmad4; lanes 14-19) were included in binding reactions to supershift complexes containing Smad3 and 4 (marked by * and **, respectively). A negative control antibody (+IgG; lanes 2-7) was used to show specificity of Smad binding. Depicted is a representative EMSA of two replicates, and fold changes were calculated from band intensities determined with Image Quant 5.0 (GE Healthcare).

In the presence of a Smad4 antibody, strong supershifts were observed (Fig. 1A, **, lanes 14-19). Smad4 bound under control conditions and progressively increased 2.6-fold over 20 hrs (compare lanes 14 and 16). The same progressive increase in Smad4 binding

(2.6-fold) was also observed in the presence of activin (lanes 17-19), but unlike Smad3, Smad4 was minimally responsive to activin. Relative to control, activin only increased Smad4 binding by 25 % at all time points (lanes 14-16 vs. 17-19). In the absence of nuclear extracts there was no binding (lane 1), and no supershifts occurred in the presence of IgG (lanes 2-7) confirming the specificity of complex formation, and Smad3 and 4 binding, respectively. These data showed that endogenous Smad3 and 4 from LβT2 cells can bind to a palindromic SBE and that Smad3, but not Smad4 binding, was transient and activin-dependent. The progressive increase in Smad4 binding over time was consistent with the induction of o*FSHBLuc*, where maximal binding and expression are similar.

Creation of a palindromic SBE (T164C) was necessary to observe Smad3 and 4 binding properties. It was thought, however, that the findings presented in Fig. 1A may not correspond to what occurs on the wild-type o*FSHB* promoter under basal and activin-stimulated conditions. Therefore, a competition EMSA was performed to see if Smad4 can bind to wild-type o*FSHB* since only Smad4 binding mimics expression of o*FSHBLuc*. This was performed at 20 hrs, which is the point of maximum Smad4 binding (Fig 1A, lanes 16 and 19). Consistent with Fig. 1A, Smad4 bound to T164C strongly under control conditions, and was unaffected by activin in this case (Fig 1B, **, lanes 4 and 5). This binding was specific because no binding or supershifts were observed in the absence of nuclear extracts (lane 1) or presence of IgG (lanes 2 and 3), respectively. Next, the ability of various unlabeled oligos to compete for Smad4 binding was examined (lanes 6-9). In lanes 6 and 7, 100-fold excess of unlabeled T164C and a palindromic SBE (SBE, Table 1), respectively, was sufficient to compete for 100 % of bound Smad4 (Fig. 1B). The same amount of a wild-

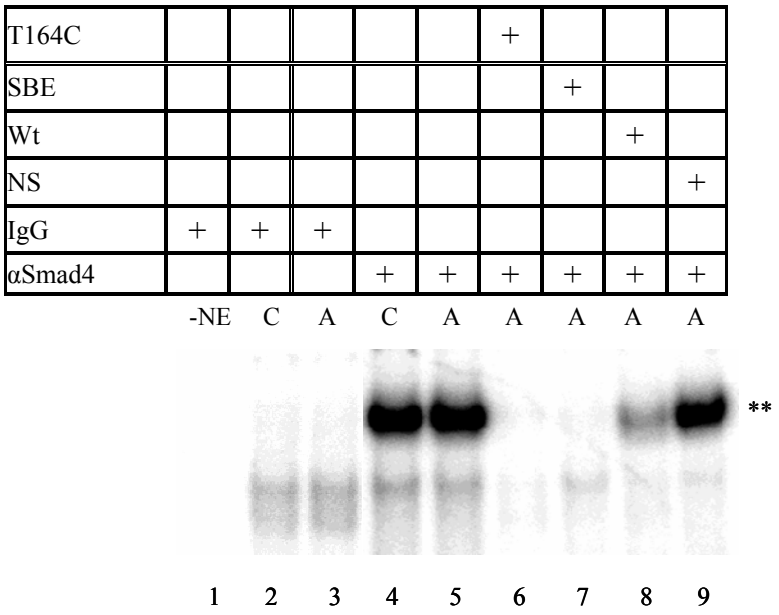


Figure 1B: Wild-type o*FSHB* can bind Smad4. A representative EMSA that used no nuclear extracts (-NE, lane 1) or LβT2 nuclear extracts that were treated with control media (C) or activin (A) for 20 hrs. These were incubated with ^{32}P-labeled T164C and an antibody to Smad4 (+αSmad4, lanes 4-9) to supershift Smad4-containing complexes (**). For competition studies, 100-fold excess of unlabeled T164C (lane 6), palindromic SBE (SBE, lane 7), wild-type o*FSHB* (Wt, lane 8) or non-specific DNA (NS, lane 9), was incubated with LβT2 nuclear extracts prior to the addition of antibody and probe. A negative control antibody (+IgG; lanes 2 and 3) was used to show specificity of Smad4 binding. Depicted is a representative EMSA of two replicates, and percent competition was calculated from band intensities determined with Image Quant 5.0.

type o*FSHB* oligo (Wt, Table 1) competed 85 % (lane 8). The specificity of competition was shown through the use of a non-specific oligo (NS, Table 1), which did not compete for the level of Smad4 bound. This suggests, for the first time, that Smad4 can bind to the single-copy SBE in the o*FSHB* promoter (-162AGAC-159) that is critical for activin induction in LβT2 cells (26, 27).

II. Smad4, but not Smad2 is required for activin induction of o*FSHBLuc* in LβT2 cells

To investigate the functional importance of Smad4 in mediating o*FSHB* induction by activin, LβT2 cells were cotransfected with o*FSHBLuc* and either empty DNA (pCMV) or

a dominant negative (DN) inhibitor of Smad4 (Smad4 1-514). After 22 hrs, activin induced o*FSHBLuc* by 5.6-fold (Fig. 2A). In the presence of the DN-Smad4, the induction was only 2.1-fold, a reduction of almost 63 %, and basal expression was unaffected (Fig. 2A).

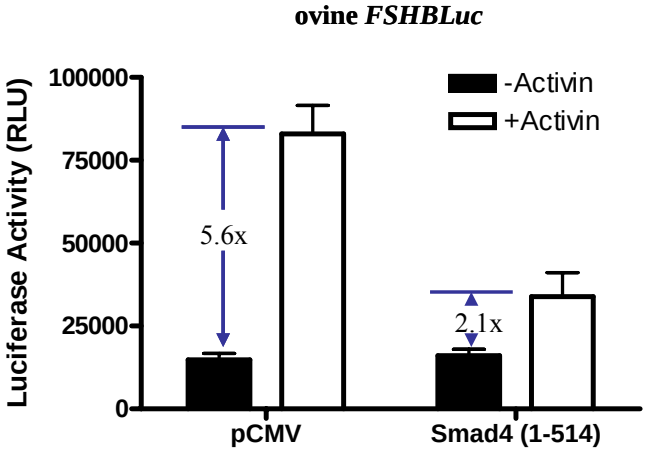
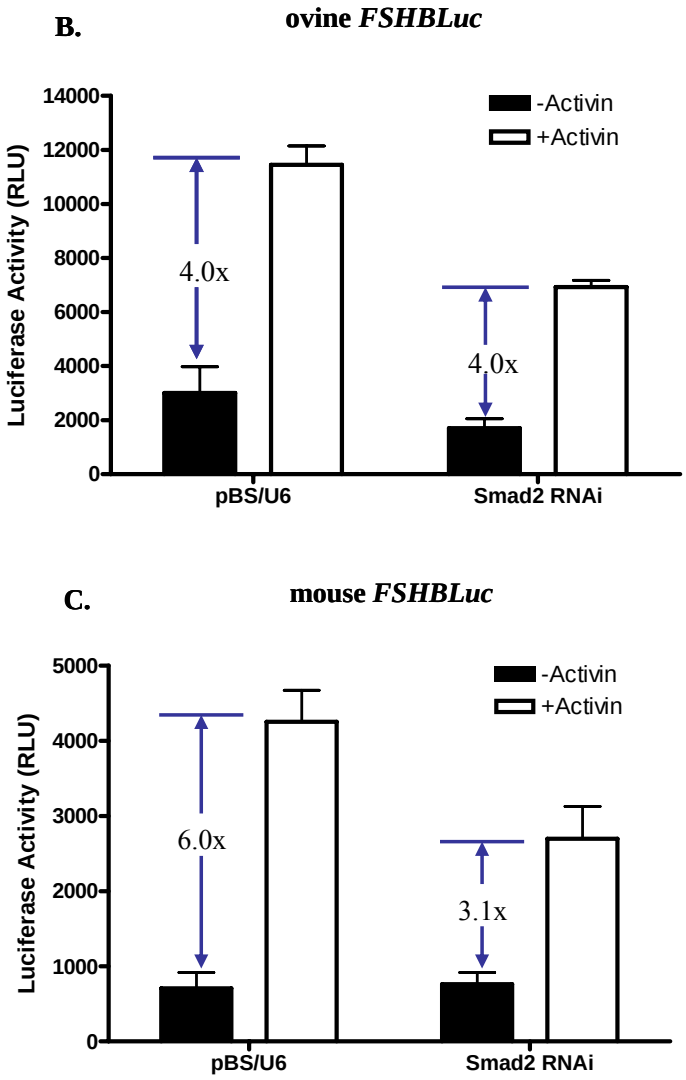


Figure 2A: Smad4 is required for activin induction of o*FSHBLuc*. As described in Materials and Methods, LβT2 cells were transiently cotransfected with o*FSHBLuc* and either empty DNA (pCMV) or a DN-Smad4 (Smad4 1-514). After 24 hrs, cultures were treated with (+Activin) or without (-Activin) 50 ng/ml of activin A for 22 hrs, and assayed for luciferase activity. The luciferase activity is plotted as mean +/- SEM, which was determined with Prism version 4 (GraphPad Software, Inc., San Diego, CA), of triplicate assays. The level of activin-induced increases in o*FSHBLuc* expression is shown as fold change and highlighted by blue arrows.

Activin induction of o*FSHBLuc* is known to occur independently of receptor-mediated Smad3 activation (see Chapter II, [23]), but the role of Smad2 remained untested. To determine if Smad2 was involved in activin induction of o*FSHBLuc*, an RNA interference (RNAi) approach was used to knockdown endogenous levels of Smad2 in LβT2 cells. A DN-Smad2 approach was not used since RNA produced from Smad2 expression vectors is degraded in LβT2 cells (16). LβT2 cells were cotransfected with o*FSHBLuc* and either a Smad2 small-interfering RNA (siRNA)- expressing construct (Smad2 RNAi) or its empty vector (pBS/U6).



Figures 2B and 2C: Smad2 is not involved in activin induction of o*FSHBLuc*. LβT2 cells were transiently transfected with o*FSHBLuc* (B) or m*FSHBLuc* (C), and simultaneously cotransfected with a Smad2 siRNA (Smad2 RNAi) or its empty vector (pBS/U6). After 24 hrs, cultures were treated with (+Activin) or without (-Activin) 50 ng/ml of activin A for 22 hrs, and assayed for luciferase activity. The luciferase activity is plotted as mean +/- SEM, which was determined with Prism version 4, of triplicate assays. The level of activin-induced increases in o*FSHBLuc* expression is shown as fold change and highlighted by blue arrows.

This Smad2 RNAi was previously documented to effectively block Smad2 signaling in LβT2 cells (16). In the presence of pBS/U6, activin induced an approximate 4-fold

increase in o*FSHB* expression (Fig. 2B). Cotransfection with the Smad2 RNAi reduced basal expression by 43 %, but the level of induction by activin at 22 hrs remained the same at 4-fold over basal (Fig. 2B). The inability of Smad2 RNAi to alter the overall magnitude of activin-induced o*FSHBLuc* expression could be attributed to the Smad2 siRNA vector being non-functional, but the decrease in basal expression of o*FSHBLuc* suggests otherwise. Nevertheless, to ensure that the Smad2 siRNA was effective in blocking activin-dependent Smad responses a mouse *FSHB* promoter/reporter (m*FSHBLuc*) was used. The Smad2 RNAi is known to block m*FSHBLuc* induction by about 50 % (16). Activin induced m*FSHBLuc* expression by 6-fold when it was cotransfected with the empty vector pBS/U6 (Fig. 2C). The Smad2 RNAi blocked almost 50 % of activin induction without affecting basal activity (Fig. 2C). Therefore, under these conditions, the Smad2 siRNA is functional and effective in abrogating activin action in LβT2 cells. Collectively, the data presented in Fig. 2A-C indicate that Smad4, but not Smad2 is required for activin induction of o*FSHB*.

III. p38 mediates the second phase of activin-induced o*FSHB* expression

Previously, pharmacological inhibition of TAK1 and one of its downstream targets, p38, revealed that these two MAPK pathway members are mediators required for transcription of o*FSHB* in response to activin (see Chapter II, [23]). These studies were conducted when activin induction of o*FSHBLuc* was highest (24 hrs), so we next sought to determine when p38 was required during the time-course of activin-induced expression of o*FSHB*. This type of analysis would indicate whether or not o*FSHB* is a direct target of p38 action. LβT2 cells transfected with o*FSHBLuc* and stimulated with activin elicited progressively increasing levels of expression over time with a maximal induction of almost

13-fold at 24 hrs (Fig. 3, solid red line). In the presence of the selective p38 inhibitor, SB203580, the same as that used in Fig. 8C of Chapter II, induction of o*FSHBLuc* from 0 to 8 hrs did not differ from the increased expression caused by activin alone (Fig. 3).

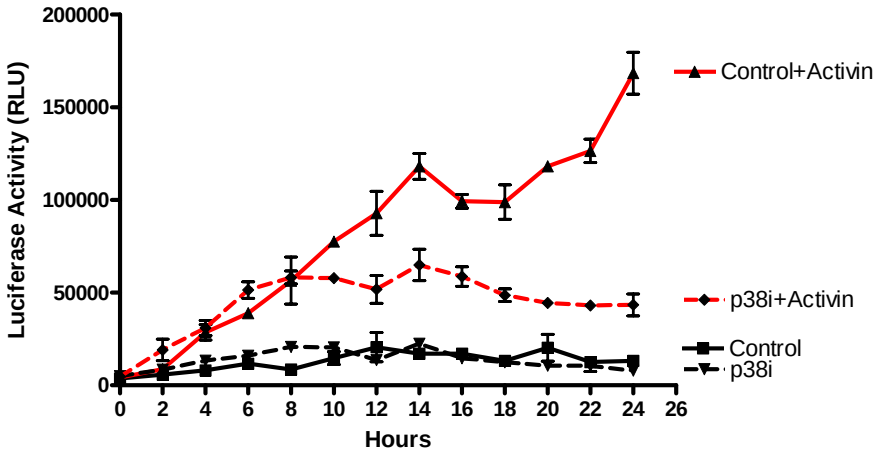


Figure 3: p38 is required for the second phase of activin-induced expression of o*FSHB* in LβT2 cells. LβT2 cells were transiently transfected with o*FSHBLuc*. After 24 hrs, cultures were pretreated for 1 hr with 50 μM of the p38 inhibitor, SB203580 (p38i), or its vehicle, DMSO (Control), so that each contained DMSO at a final concentration of 0.1 %. Cultures were then treated with activin A at 50 ng/ml in the presence of Control (solid red line) or p38i (dashed red line), or without activin A in the presence of Control (solid black line) or p38i (dashed black line). Cells were lysed every 2 hrs for 24 hrs and samples were assayed for luciferase activity. Data are plotted as mean luciferase activity +/- SEM, which was determined with Prism version 4, of triplicate assays.

After 8 hrs, however, the p38 inhibitor prevented any further increases in o*FSHB* expression, and maintained the level of activin induction over the subsequent 16 hrs at a consistent level, averaging 3.7 +/- 0.3-fold (Fig. 3). The data presented in Fig. 3 indicate that the induction of o*FSHB* by activin occurs in biphasic manner, and that p38 is necessary for the second phase, which occurs after 8 hrs and is responsible for 50 % of overall o*FSHB* induction.

IV. *FOXQ1* mRNA is induced transiently by activin

Due to the physiological importance of a putative Runx1/FOX binding site in mediating activin induction of o*FSHB* (see Chapter III, [25]), the *FOX* family was examined to determine if any of its 43 members (34) are regulated by activin. The rationale for this is based upon the fact that o*FSHB* is a late-responding gene that is induced biphasically (Fig. 3), so its expression is likely controlled by another activin-activated and early-responding gene. The *Runx* family was not investigated because they are typically regarded as constitutively expressed genes. One candidate gene, *FOXQ1*, was identified based on a

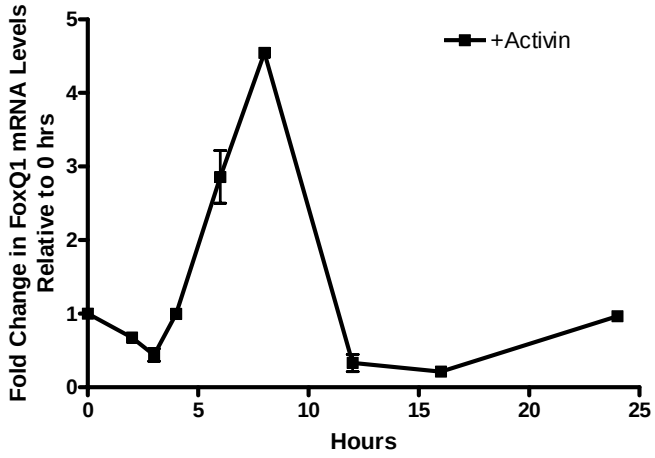


Figure 4: Endogenous *FOXQ1* mRNA is transiently upregulated by activin in LβT2 cells. LβT2 cells were treated with activin for the indicated times, RNA was isolated and quantitative RT-PCR was performed as described in Materials and Methods. Threshold cycle (Ct) values were normalized to endogenous mouse 18s ribosomal RNA, then used to quantitate *FOXQ1* mRNA expression with the $2^{-\Delta\Delta Ct}$ method for quantiation (35). The mRNA levels were quantiated relative to the mRNA levels of *FOXQ1* at 0 hrs, which was normalized to 1. Plotted are the means +/- SEM, which was determined with Prism version 4, of triplicate assays.

single DNA microarray that showed a 2.2-fold increase in *FOXQ1* mRNA expression in LβT2 cells after 2 hrs of activin stimulation (data not shown). No other *FOX* members were

regulated by activin within this timeframe (data not shown). Regulation of *FOXQ1* by activin was investigated further through quantitative RT-PCR (qRT-PCR). Depicted in Fig. 4 are time-course data for activin-induced changes in endogenous *FOXQ1* mRNA levels in LβT2 cells. Relative to *FOXQ1* mRNA (*foxq1*) levels at 0 hrs, activin increased expression of *foxq1* mRNA by almost 3-fold at 6 hrs, and a maximum induction of 4.5-fold occurred at 8 hrs (Fig. 4). After 8 hrs, the activin-induced expression of *FOXQ1* mRNA quickly declined and, on average, returned to a basal level (Fig. 4). Although an increase in *foxq1* was not seen at 2 hrs, as suggested by the microarray, it was found to be regulated by activin in an early and transient fashion.

DISCUSSION

I. Defining the role of Smads in activin induction of o*FSHB*

Activin is well known to be a potent inducer of *FSHB* transcription and FSH secretion (2, 3, 35). One classical intracellular pathway utilized by activin is through the phosphorylation of Smad2 and/or Smad3, followed by oligomerization with Smad4, then nuclear translocation. The heteromeric Smad complex then regulates gene expression by binding to SBEs. Activin induction of mouse and rat *FSHB* in LβT2 cells is dependent on Smads 2 and 3 due to the existence of an 8 bp palindromic SBE, located between -266 bp and -259 bp that is unique to rodent promoters (16-19). The role of Smads in activin-stimulated transcription of o*FSHB*, which is highly homologous to human and porcine *FSHB* has remained elusive.

Two putative single-copy (4 bp) SBEs, with starting positions at -162 bp and -134 bp, are required for activin induction of o*FSHBLuc* in LβT2 cells (24, 25). This indicates that Smads may be involved in the transcriptional response. The SBE at -134 bp has been suggested to bind Smad4 under basal conditions (24), but this interaction was not convincing, nor did it implicate Smad4 as a mediator of the activin response. To date, no Smads have been shown to bind the SBE at -162 bp under basal (24) or activin-stimulated conditions in any species.

A. Smad4, but not Smad3 binds a palindromic SBE in increasing levels over time

Because the single-copy SBE at -162 bp is necessary for 68 % of activin induction of o*FSHBLuc* in LβT2 cells (25), we first investigated whether or not Smads 3 and 4 bound to this element. To do this, it was necessary to create a palindromic SBE, spanning from -166

bp to -159 bp of the o*FSHB* promoter (T164C, Table 1). In EMSA, ^{32}P-labled T164C bound to LβT2 nuclear proteins in an activin-dependent and transient fashion (Fig. 1A, lanes 2-7), and these complexes were found to contain both Smad3 and 4 (lanes 11-19). The transient level of activin induced binding was reflected directly in the amount of Smad3 bound to T164C (lanes 11-13), and correlated with the rapid phosphorylation and nuclear translocation of Smad3 in response to activin in LβT2 cells (16). It also followed the pattern of p3TPLuc induction in LβT2 cells, but not with the kinetics of activin induced o*FSHBLuc* expression (see Chapter II, [23]). Importantly, Smad4 binding was highest at 20 hrs (lanes 16 and 19), which does follow the kinetics of o*FSHBLuc* induction.

B. Smad4 binds the -162 bp SBE in the wild-type o*FSHB* promoter

Although it was necessary to create an artificial o*FSHB* probe that contained an 8 bp SBE to observe the properties of Smad3 and 4 binding, through a competition EMSA we were able to show for the first time that Smad4 can bind to the single-copy SBE at -162 bp (Fig. 1B). A lack of observable binding to the wild-type o*FSHB* promoter could be attributed to either lower affinity of the single-copy SBE for Smads, or instability of protein:DNA complexes during electrophoresis. The former is more appropriate, however, given that the Wt o*FSHB* oligo competes for most (85 %), but not all of the Smad4 bound to T164C under activin-stimulated conditions (Fig. 1B, lane 8). Collectively, the data presented in Fig. 1 indicate that Smad4 binds the o*FSHB* promoter and may be important for its synthesis.

C. Smad3 is not important for induction of o*FSHB* by activin

Based on the pattern of activin-stimulated Smad3 binding (Fig. 1A), it does not seem important for activin induction of o*FSHB*. This is consistent with our previous finding that

receptor-mediated Smad3 phosphorylation is not required for activin induction of o*FSHB* (see Chapter II, [23]). If Smad3 were important, then maximal binding and nuclear availability would likely occur at 20 hrs, not at 1 and 4 hrs when activin induction of o*FSHB* is almost non-existent.

D. Smad4, but not Smad2 mediates activin induction of o*FSHBLuc*

Figure 2A of this study showed that Smad4 is important for activin induction of o*FSHBLuc* because a DN-Smad4 (Smad4 1-514) blocked 63 % of the activin response. This DN-Smad4 was designed based on a naturally occurring Smad4 mutation that has a C-terminal truncation of 38 amino acids that is present in pancreatic adenocarcinomas (36). This prevents the formation of transcriptionally active DNA-binding complexes, such as Smad2/4 (37). Even though Smad2 cannot directly contact DNA, it can be targeted to promoters by Smad4 to regulate transcription. However, through an RNAi approach, Smad2 was not found to be involved in the induction of o*FSHBLuc* by activin in LβT2 cells (Fig. 2B). Therefore, induction of o*FSHB* expression in LβT2 cells likely depends on an alternate, activin-activated transcription factor that associates with Smad4.

E. A Smad4 partner protein may be important for activin induction of o*FSHB*

Primary support for the dependence of o*FSHB* induction on a Smad4-associated transcription factor is based upon the inhibitory actions of DN-Smad4 (Fig. 2A). In the presence of the DN-Smad4, a transcription factor important for activin's actions would be sequestered away from the promoter. In addition, binding of Smad4 to the single-copy SBE at -162 bp in the o*FSHB* promoter is weaker than for the palindromic SBE (Fig. 1B), so the additional factor may act to stabilize or enhance the interaction between Smad4 and DNA,

and provide specificity to the -162 bp SBE in the o*FSHB* promoter. Furthermore, Smad4 is a common mediator (Co-Smad) that is not regarded as an independent transcription factor, nor is it regulated by activin. Consistent with the former, overexpression of Smad4 alone in LβT2 cells cannot enhance the levels of basal or activin-stimulated expression of rodent (16-18) or ovine (personal observation) *FSHB*. Regarding the latter, Smad4 binding was not found to be activin-dependent (Figs. 1A and B). However, given the sheer number of transcription factors that can interact with Smad4 (15), identification of this factor is not a trivial undertaking. Nevertheless, we have shown that Smad4 (Fig. 2A), and not Smad2 (Fig. 2B) or 3 (see Chapter II, [23]) is an important mediator of activin induced o*FSHB* expression.

II. The role of p38 in activin induction of o*FSHB*

Although we defined the importance of Smad4 in o*FSHB* regulation, it was also important to investigate the MAPK pathway since we previously discovered that TAK1 and its downstream target, p38, are necessary for activin-induced expression of o*FSHBLuc* in LβT2 cells (see Chapter II, [23]). In Fig. 3 of this study, a time-course approach was adopted to examine the role of p38 in greater detail. Perhaps one of the most interesting discoveries reported in this study was the finding that through pharmacological inhibition of p38, the induction of o*FSHBLuc* by activin could be separated into two phases (Fig. 3). The second phase, which occurred after 8 hrs, required p38, whereas the first phase was p38-independent (Fig. 3). This confirmed our previous data, but expanded it by showing that o*FSHB* is an indirect target of p38 because even in the presence of 50 μM SB203580, induction continued up to 8 hrs (Fig. 3). This timeframe is when transcriptional responses directly activated by

p38 occur. For example, direct actions of p38 are required for TGFβ-mediated regulation of the angiotensin II type I receptor (38) and thrombospondin-1 (39) genes. Both are significantly induced by TGFβ within 4 hrs and return to basal after 12 hrs. These two examples are typical models of the rapid and transient responses mediated by p38 (29) that are clearly different from the slow induction of o*FSHB* (Fig. 3).

III. Rationale for a transcriptionally induced Smad4 partner

The data presented in Fig. 3 also supported our hypothesis that o*FSHB* synthesis likely depends on an activin-regulated early response gene. This is not based simply upon the fact that its kinetics are slow relative to classical p38 and Smad transcriptional events, but because activin induction of o*FSHBLuc* was found to be a two-step process (Fig. 3). The early response gene could ultimately be necessary for the maximal level of o*FSHB* induction during the latter phase through a "self-enabling" mechanism. Self-enabling transcription occurs when a ligand induces a gene rapidly, which itself is a transcription factor that can regulate the original action of the ligand through a secondary gene response. This mechanism of gene regulation is not uncommon to TGFβ family signaling (40). One example of a self-enabling mechanism occurs in endothelial cells. Rapid, Smad-dependent induction of *ATF3* by TGFβ leads to subsequent regulation of *Id1* (inhibitors of differentiation 1) expression by an interaction between ATF3 and Smad3 (41). Similarly, in Xenopus embryos, *Mixer* is induced by activin, and its product binds the *goosecoid* promoter and enhances its transcription in conjunction with Smads 2 and 4 (42). A similar mechanism may exist to control activin-mediated induction of o*FSHB* expression, and one possible candidate was identified in this study (FOXQ1).

IV. FOXQ1 as a possible inducer of o*FSHB* transcription in LβT2 cells

A. FOXQ1 may bind the o*FSHB* promoter in association with Smad4

Depicted in Fig. 4 are data showing the pattern of activin induction of *FOXQ1* in LβT2 cells. *FOXQ1* may be an early response gene important for driving o*FSHB* transcription in a self-enabling manner. Our discovery here was novel, not only because no *FOX* members have been found to be transcriptionally regulated by activin in LβT2 cells, but also because of the physiological importance of a putative FOX binding site juxtaposed upstream to the -162 bp single-copy SBE (see Chapter III, [25]). The maximum induction of *FOXQ1* occurred between 6 hrs and 8 hrs (Fig. 4). After this period, its product, FOXQ1, may partner with Smad4. The FOXQ1/Smad4 complex could bind the putative FOX and Smad binding sites between -171 bp and -159 bp of the o*FSHB* promoter, driving the secondary and highest increase in o*FSHB* synthesis. This would generate classical "self-enabling" transcription and may be possible for several reasons. The first is that Smads are well known to interact with FOX proteins (15, 26-28). In addition, the abundance of Smad4 bound is highest at 20 hrs (Fig. 1A and 1B), and Smad4 alone cannot transactivate *FSHB* (see above, section I.E). The FOXQ1/Smad4 partnership may also explain why Smad4, but not Smad2 or 3, is important for activin induction of o*FSHBLuc*.

B. Mechanisms of *FOXQ1* regulation

We observed that *FOXQ1* regulation by activin is both rapid and transient (Fig. 4), correlating with the kinetics of genes directly regulated by p38 and Smads. Therefore, it is possible that in LβT2 cells, *FOXQ1* is rapidly upregulated in response to activin through direct actions of Smad- and/or p38-dependent mechanisms. In epithelial cells, *FOXQ1*

expression is altered by TGFβ at 6 hrs in a Smad4-dependent manner (43). Although *FOXQ1* is inhibited in these cells (43), this study demonstrated that *FOXQ1* can be rapidly regulated by TGFβ through Smads. Because activin and TGFβ can elicit opposite effects, such as with mouse *FSHB* (see Chapter I, [22]), it was not unusual that we saw stimulation of *FOXQ1*, while others have reported repression. The precise mechanisms responsible for activin regulation of *FOXQ1* in LβT2 cells, however, are currently unknown and deserve further investigation.

C. Evidence against Runx proteins as inducers of o*FSHB*

Although we identified FOXQ1 as a candidate Smad4-associated mediator of activin-induced o*FSHB* expression, it is important to address the Runx family (Runx1-3) of transcription factors. These are also known to interact with Smads (26) and bind to DNA sequences similar to those for FOX proteins (27). A Runx protein could therefore be involved in o*FSHB* regulation by associating with Smad4 and binding the putative FOX (Runx) site that we previously identified (see Chapter III, [25]). We did not investigate the regulation of Runx members because they are generally expressed at constitutive levels, and activin-induced o*FSHB* expression may rely on a transcriptionally activated gene (see sections III and IV.A). There are some reports of Runx regulation, however.

Bone morphogenetic proteins (BMPs) have been reported to increase *Runx2* expression in osteoblasts and myoblasts (44-46). In addition, the MAPK pathway has been shown to phosphorylate Runx2 (47). It is unknown if Runx2 phosphorylation is ligand-dependent because this was discovered using overexpressed, constitutively active MAPK. These transcriptional and post-translational regulatory events occurred in osteoblasts. Runx

is critical for osteoblast differentiation and bone formation, as shown in *Runx2* deficient mice (48, 49). Thus, the importance of Runx seems to be in regulating skeletal development. Restricting Runx activity to bone cells could be a physiological mechanism to distinguish between two different families of transcription factors (Runx and FOX) that bind similar DNA sequences. Therefore, FOX proteins, such as FOXQ1, may be more important in regulating pituitary functions, such as activin-induced transcription of o*FSHB*.

D. FOXL2 is unlikely involved in activin induction of o*FSHB*

In support of the role of FOX proteins in the pituitary, another member, FOXL2, is expressed in the pituitary (50) and seems to have a functional role. In conjunction with Smads and AP-1, FOXL2 binds to the GnRH receptor activating sequence (GRAS) (51). GRAS is a sequence in the GnRH receptor gene (*GnRH-R*) that is required for its induction by activin (53). The role of FOXL2, however, seems to reside in early pituitary development since its expression precedes that of most gonadotrope markers (52). Consistent with this, induction of *GnRH-R* by activin occurs in αT3-1 cells (51, 53), a cell line thought to represent fetal gonadotropes. The αT3-1 cell line does not express *FSHB* or induce o*FSHBLuc* in response to activin (54). This suggests that FOXL2 may not be involved in activin-dependent transcription of o*FSHBLuc* because FOXL2 and Smads can mediate an activin response in the gonadotrope cell line, αT3-1 (51, 53). Because αT3-1 cells are fetal gonadotropes, they may lack other signaling components that are present in LβT2 cells. The absence of these unknown components could explain the lack of activin-induced o*FSHB* induction in αT3-1 cells. Nevertheless, FOXL2 is present constitutively and could immediately induce o*FSHBLuc* in LβT2 cells in conjunction with Smads. Therefore,

FOXQ1 may be more relevant to transcription of o*FSHBLuc* since we identified it as an early-responding and activin-regulated gene in LβT2 cells (Fig. 4), but more studies are necessary to characterize the role of FOXQ1 further (see Concluding Remarks).

CONCLUDING REMARKS

I. Dissertation summary

The preceding chapters of this dissertation have aided significantly in understanding the complexity surrounding activin-mediated transcription of *FSHB*. The studies in Chapter I showed not only that activin and TGFβ have opposing actions on m*FSHB* expression in primary mouse gonadotropes (Chapter I, Fig. 7A), but indicated that *FSHB* transcription likely depended on a transcription factor other than Smad2 and 3, which can both be activated by activin and TGFβ. In agreement with this hypothesis, Chapter II showed that receptor-mediated activation of Smad3 was not important for activin induction of o*FSHB* and that the kinetics of o*FSHB* induction were atypical of a Smad-dependent response (Chapter II, Fig. 3). In Chapter III, we identified and showed the physiological importance of a putative FOX/Runx binding site juxtaposed to a SBE (Chapter III, Fig. 2). This finding was important for two reasons. First, it indicated that either Smad2 or 4 may be involved in o*FSHB* synthesis because the SBE at -162 bp is required for its induction by activin in LβT2 cells (Chapter III, Fig. 2). Second, it narrowed the search for an alternate and important transcription factor to two families, FOX and Runx, both of which contain members known to interact with Smads. FOX proteins, however, seemed more relevant since Runx activity seems restricted to bone formation.

Importantly, Chapter IV expanded these studies by showing that Smad4 is capable of binding the -162 bp single-copy SBE (Chapter IV, Fig. 1B) and that Smad4, but not Smad2 is important for o*FSHB* induction (Fig. 2). Moreover, we found that activin induction of o*FSHBLuc* occurs biphasically, with only the second phase requiring p38 (Fig. 3), suggesting

the involvement of a rapidly activated transcription factor gene that would be required for the second phase of o*FSHB* induction. Our identification of one gene, *FOXQ1*, was a logical candidate given its kinetics (Fig. 4), the importance of a putative FOX binding site in the o*FSHB* promoter upstream to a SBE that binds Smad4 (Fig. 1B), and the inability of Smad4 alone to activate ovine (data not shown), as well as rodent *FSHB* (16-18) expression. As previously mentioned, it is possible that FOXQ1 may bind to the site we identified in conjunction with Smad4 to regulate activin-dependent transcriptional activity of o*FSHB*, but this is a hypothesis that needs further attention. Therefore, it is important to address some remaining areas of exploration.

II. Future Directions

A. Investigating FOXQ1 binding to the o*FSHB* promoter

One important study would be to demonstrate activin-dependent binding of both FOXQ1 and Smad4 to the sites spanning -171 bp and -159 bp of the o*FSHB* promoter. This would further implicate FOXQ1 as a potential mediator of activin-stimulated o*FSHB* synthesis, but is not an experiment that can be easily conducted. As previously mentioned, EMSAs could not be conducted with LβT2 nuclear extracts, either control- or activin-treated, and a ^{32}P-labeled probe encompassing -171 bp to -159 bp of the wild-type o*FSHB* promoter (Wt, Table 1). To reproducibly observe Smad:DNA interactions in EMSA, a mutation at -164 bp that created an 8 bp palindromic SBE (T164C, Table 1) was necessary. Although it enabled us to study Smad binding properties, the T164C probe may not be useful to investigate FOXQ1 binding. The T to C mutation creates a mismatch in the putative FOX binding site (Fig. 5) that may diminish the affinity of FOXQ1 for DNA. Because of this and

the inability of the Wt probe to effectively bind LβT2 nuclear extracts, EMSA would be insufficient to examine FOXQ1 binding.

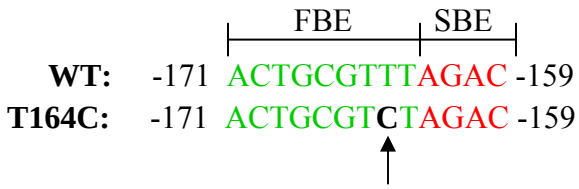


Figure 5: A mismatch in the putative FOXQ1 binding element. The wild-type (WT) o*FSHB* promoter contains a putative FOXQ1 binding element (FBE) juxtaposed to a Smad binding element (SBE). Nucleotides that may be important for FOXQ1 binding are shown in green, and those important for Smad4 binding are shown in red. Substitution of the T at -162 bp to a C, shown in bold black (arrow), creates a mutation within the putative FBE, thus decreasing the affinity of FOXQ1 for the FBE.

One possible method to circumvent this problem would be chromatin immunoprecipitation (ChIP) assays that, unlike EMSA, would not rely on small oligonucleotides and artificial buffer conditions. Furthermore, because of crosslinking in ChIP assays, low affinity protein:DNA complexes could be stabilized. Importantly, this method could be used to show *in vivo* interactions between FOXQ1, as well as Smad4, and the o*FSHB* promoter, but would require the use of a transient or stable ChIP assay. This is a procedure useful for examining interactions between nuclear proteins and transiently (55), or stably transfected genes, such as studying activin-dependent binding between transformed mouse gonadotrope (LβT2)-derived transcription factors, and ovine *FSHB*. However, another putative FOX/Runx binding site exists between -66 bp and -58 bp of the o*FSHB* promoter that may also bind FOXQ1. Therefore, it would be necessary to ensure that any interactions observed between FOXQ1 and the o*FSHB* promoter are specific to the site between -171 bp and -163 bp and not the one further downstream.

B. Investigating the role of FOXQ1 in activin induction of o*FSHB*

Another area of exploration is to examine the functional importance of FOXQ1 in activin regulation of o*FSHB*. Because FOXQ1 is only one of a 43 member protein family (34) that bind the same DNA sequences, this analysis will be critical to proving that FOXQ1 and not another FOX member is the key mediator of o*FSHB* induction. Currently, no dominant negative forms of FOXQ1 exist, so using RNAi to knockdown endogenous FOXQ would be most suitable to examine its role in LβT2 cells. However, use of this technique would require great care to ensure specificity of the RNAi to FOXQ1 because often, RNAi approaches produce several non-specific effects (reviewed in [58]).

Most importantly, the Runx family also deserves further investigation. Recently, Ruebel et al. showed that TGFβ1 upregulated Runx1 by 2-fold in the human pituitary cell line, HP75 (57), which express the gonadotrope markers, *FSHB* and *LHB* (58). We identified FOXQ1 as a potential candidate transcription factor that may be important for activin induction of o*FSHB* (Chapter IV), but Runx proteins may be important for gonadotrope function in addition to their roles in bone development. The work presented in Chapters I - IV of this dissertation have significantly advanced knowledge about the molecular mechanisms involved in activin-induced transcription of o*FSHB*. As described in this section, important and complex areas of research remain, however. The most important and novel of which would be to prove (or disprove) that FOXQ1 drives activin induction of o*FSHB*. If FOXQ1 is not functionally important, then identification of another FOX, or perhaps, Runx member will be crucial to understanding what controls *FSHB* expression and FSH production.

REFERENCES

1. Kumar, T.R., Wang, Y., Lu, N., and M.M. Matzuk. 1997. Follicle-stimulating hormone is required for ovarian follicle maturation but not male fertility. Mol. Endocrinol. 13, 851-865.

2. Dalkin, A.C., Haisenleder, D.J., Ortolano, G.A., Ellis, T.R., and J.C. Marshall. 1989. The frequency of gonadotropin-releasing-hormone stimulation differentially regulate gonadotropin subunit messenger ribonucleic acid expression. Endocrinology. 125, 917-924.

3. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo,W., Karr, D., and J. Spiess. 1986. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature. 321, 776-779.

4. Ling, N., Ying, S.Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and R. Guillemin. 1986. A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem. Biophys. Res. Comm. 138, 1129-1137.

5. Huang, H.-J., Wu, J.C., Zhirnov, O., and W.L. Miller. 2001. A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology. 142, 2275-2283.

6. Bilezikjian, L.M., Blount, A.L., Leal, A.M.O., Donaldson, C., Fischer, W., and W. Vale. 2004. Autocrine/paracrine regulation of pituitary function by activin, inhbin and follistatin. Mol. Cell Endocrinol. 225, 29-36.

7. Vale, W., Hsueh, A., Rivier, C., and J. Yu. 1990. The inhibin/activin family of growth factors. Peptide Growth Factors and Their Receptors, Handbook of Experimental Pharmacology. 211-248. Eds. M.A. Sporn and A.B. Roberts. Heidelberg: Springer-Verlag.

8. Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and S. Shimasaki. 1991. Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. Endocrinology. 129, 815-822.

9. Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fendley, B.M., Mason, A.J., Chin, W.W., Schwall, R.H., and W. Vale. 1991. Evidence for an autocrine role of activin B within rat anterior pituitary cultures. Endocrinology. 128, 1682-1684.

10. DePaolo, L.V., Shimonaka, M., Schwall, R.H., and N. Ling. 1991. In vivo comparison of the follicle-stimulating hormone-suppressing activity of follistatin and inhibin in ovariectomized rats. Endocrinology. 128, 668-674.

11. Ethier, J.F., and J.K. Findlay. 2001. Roles of activin and its signal transduction mechanisms in reproductive tissues. Reproduction. 121, 667-675.

12. Bernard, D.J., Lee, K.B., and M.M. Santos. 2006. Activin B can signal through both ALK4 and ALK7 in gonadotrope cells. Reprod. Biol. Endocrinol. 4, 52.

13. Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and S.E. Kern. 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol. Cell. 1, 611-617.

14. Shi, Y., Wang, Y.F., Jayaraman, L., Yang, H., Massague, J., and N.P. Pavletich. 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell. 4, 585-594.

15. Ross, S., and C.S. Hill. 2007. How the Smads regulate transcription. Int. J. Biochem. Cell Biol. 40, 383-408.

16. Bernard, D.J. 2004. Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone β subunit in mouse gonadotrope cells. Mol. Endocrinol. 18, 606-623.

17. Lamba, P., Santos, M.M., Philips, D.P., and D.J. Bernard. 2006. Acute regulation of murine follicle-stimulating hormone β subunit transcription by activin A. J. Mol. Endocrinol. 36, 201-220.

18. Suszko, M.I., Lo, D.J., Suh, H., Camper, S.A., and T.K. Woodruff. 2003. Regulation of the rat follicle-stimulating hormone β-subunit promoter by activin. Mol. Endocrinol. 17, 318-332.

19. Suszko, M.I., Balkin, D.M., Chen, Y., and T.K. Woodruff. 2005. Smad3 mediates activin-induced transcription of follicle-stimulating hormone β-subunit gene. Mol. Endocrinol. 19, 1849-1858.

20. Dupont, J., McNeilly, J., Vaiman, A., Canepa, S., Combarnous, Y., and C. Taragnat. 2003. Activin signaling pathways in ovine pituitary and LβT2 gonadotrope cells. Biol. Reprod. 68, 1877-1887.

21. Wu, J.C., Su, P., Safwat, N., Sebastian, J., and W.L. Miller. 2004. Rapid, efficient isolation of murine gonadotropes and their use in revealing control of follicle-stimulating hormone by paracrine pituitary factors. Endocrinology. 145, 5832-5839.

22. Gore, A.J., Philips, D.P., Miller, W.L., and D.J. Bernard. 2005. Differential regulation of follicle stimulating hormone by activin A and TGFB1 in murine gonadotropes. Reproductive Biology and Endocrinology. 3:73.

23. Safwat, N., Ninomiya-Tsuji, J., Gore, A.J., and W.L. Miller. 2005. Transforming growth factor β-activated kinase 1 is a key mediator of ovine follicle-stimulating hormone β-subunit expression. Endocrinology. 146, 4814-4824.

24. Bailey, J.S., Rave-Harel, N., McGillivray, S.M., Coss, D., and P.L. Mellon. 2004. Activin regulation of the follicle-stimulating hormone β-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol. Endocrinol. 18, 1158-1170.

25. Su, P., Shafiee-Kermani, F., Gore, A.J., Jia, J., Wu, J.C., and W.L. Miller. 2007. Expression and regulation of the β-subunit of ovine follicle-stimulating hormone relies heavily on a promoter sequence likely to bind Smad-associated proteins. Endocrinology. 148, 4500-4508.

26. Jakubowiak, A., Pouponnot, C., Berguido, F., Frank, R., Mao, S., Massague, J., and S.D. Nimer. 2000. Inhibition of the transforming growth factor β1 signaling pathway by the AML1/ETO leukemia-associated fusion protein. J. Biol. Chem. 275, 40282-40287.

27. Ji, C., Eickelberg, O., McCarthy, T.L., and M. Centrella. 2001. Control and counter-control of TGF-β activity through FAST and Runx (CBFa) transcriptional elements in osteoblasts. Endocrinology. 142, 3873-3879.

28. Attisano, L., Silvestri, C., Izzi, L., and E. Labbe. 2001. The transcriptional role of Smads and FAST (FoxH1) in TGFβ and activin signaling. Mol. Cell Endocrinol. 180, 3-11.

29. Karin, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. 270, 16483-16486.

30. Huang, H.-J., Sebastian, J., Strahl, B.D., Wu, J.C., and W.L. Miller 2001. The promoter for the ovine follicle-stimulating hormone-beta gene (FSHbeta) confers FSHbeta-like expression on luciferase in transgenic mice: regulatory studies in vivo and in vitro. 142, 2260-2266.

31. Lagna, G., Hata, A., Hemmati-Brivanlou, A., and J. Massague. 1996. Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. Nature. 383, 832-836.

32. Andrews, N.C., and D.V. Faller. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Research. 19, 2499.

33. Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 25, 402-408.

34. Katoh, M., and M. Katoh. 2004. Human FOX gene family (review). Int. J. Oncology. 25, 1495-1500.

35. Weiss, J., Guendner, M.J., Halvorson, L.M., and J.L. Jameson. 1995. Transcriptional activation of the follicle-stimulating hormone beta-subunit gene by activin. Endocrinology. 136, 1885-1891.

36. Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H., and S.E. Kern. 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science. 271, 350-353.

37. Maurice, D., Pierreux, C.E., Howell, M., Wilentz, R.E., Owen, M.J., and C.S. Hill. 2001. Loss of Smad4 function in pancreatic tumors: C-terminal truncation leads to decreased stability. J. Biol. Chem. 276, 43175-43181.

38. Martin, M.M., Buckenberger, J.A., Jiang, J., Malana, G.E., Knoell, D.L., Feldman, D.S., and T.S. Elton. 2007. TGFβ1 stimulates human AT_1 receptor expression in lung fibroblasts by cross talk between the Smad, p38 MAPK, JNK, and PI3K signaling pathways. Am. J. Physiol. Lung Cell. Mol. Physiol. 293, 790-799.

39. Nakagawa, T., Lan, H.Y., Glushakova, O., Zhu, H.J., Kang, D.-H., Schreiner, G.F., Bottinger, E.P., Johnson, R.J., and Y.Y. Sautin. 2005. Role of ERK1/2 and p38 mitogen-activated protein kinases in the regulation of thrombospondin-1 by TGFβ1 in rat proximal tubular cells and mouse fibroblasts. J. Am. Soc. Nephrol. 16, 899-904.

40. ten Dijke, P., and C.S. Hill. 2004. New insights into TGFβ-Smad signalling. Trends Biochem. Sci. 29, 265-273.

41. Kang, Y., Chen, C.-R., and J. Massague. 2003. A Self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in endothelial cells. Mol. Cell. 11, 915-926.

42. Germain, S., Howell, M., Esslemont, G.M., and C.S. Hill. 2000. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. Genes Dev. 14, 435-451.

43. Levy, L., and C.S. Hill. 2005. Smad4 dependency defines two classes of transforming growth factor β (TGFβ) target genes and distinguishes TGF-β-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. Mol. Cell. Biol. 25, 8108-8125.

44. Tsuji, K., Ito, Y., and M. Noda. 1998. Expression of the PEBP2alphaA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone. 22, 87-92.

45. Tou, L., Quibria, N., and J.M. Alexander. 2003. Transcriptional regulation of the human Runx2/Cbfa1 gene promoter by bone morphogenetic protein-7. Mol. Cell. Endocrinol. 205, 121-129.

46. Banerjee, C., Javed, A., Choi, J.-Y., Green, J., Rosen, V., van Wijnen, A.J., Stein, J.L., Lian, J.B., and G.S. Stein. 2001. Differential regulation of the two principle Runx2/Cbfa1 n-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. Endocrinology. 142, 4026-4039.

47. Xiao, G., Jiang, D., Thomas, P., Benson, M.D., Guan, K., Karsenty, G., and R.T. Franceschi. 2000. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J. Biol. Chem. 275, 4453-4459.

48. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and T. Kishimoto. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 89, 755-764.

49. Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B., and M.J. Owen. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell. 89, 765-771.

50. Treier, M., Gleiberman, A.S., O'Connell, S.M., Szeto, D.P., McMahon, J.A., McMahon, A.P., and M.G. Rosenfield. 1998. Multistep signaling requirements for pituitary organogenesis in vivo. Genes Dev. 12, 1691-1704.

51. Ellsworth, B.S., Burns, A.T., Escudero, K.W., Duval, D.L., Nelson, S.E., and C.M. Clay. 2003. The gonadotroping releasing hormone (GnRH) receptor activating sequence (GRAS) is a composite regulatory element that interacts with multiple classes of transcription factors including Smads, AP-1 and a forkhead DNA binding protein. Mol. Cell. Endocrinol. 206, 93-111.

52. Ellsworth, B.S., Egashira, N., Haller, J.L., Butts, D.L., Cocquet, J., Clay, C.M., Osamura, R.Y., and S.A. Camper. 2006. FOXL2 in the pituitary: molecular, genetic, and developmental analysis. Mol. Endocrinol. 20, 2796-2805.

53. Norwitz, E.R., Xu, S., Jeong, K., Bedecarrats, G.Y., Winebrenner, L.D., Chin, W.W., and U.B. Kaiser. 2002. Activin A augments GnRH-mediated transcriptional activation of the mouse GnRH receptor gene. Endocrinology. 143, 985-997.

54. Pernasetti, F., Vasilyev, V.V., Rosenberg, S.B., Bailey, J.S., Huang, H.-J., Miller, W.L., and P.L. Mellon. 2001. Cell-specific transcriptional regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell mode. Endocrinology. 142, 2284-2295.

55. Lavrrar, J.L., and P.J. Farnham. 2004. The use of transient chromatin immunoprecipitation assays to test models for E21-specific transcriptional activation. J. Biol. Chem. 279, 46343-46349.

56. Svoboda, P. 2007. Off-targeting and other non-specific effects of RNAi experiments in mammalian cells. Curr. Opin. Mol. Ther. 9, 248-257.

57. Ruebel, K.H., Leontovich, A.A., Tanizaki, Y., Jin, L., Stilling, G.A., Zhang, S., Coonse, K., Scheithauer, B.W., Lombardero, M., Kovacs, K., and R.V. Lloyd. 2008. Effects of TGFbeta1 on gene expression in the HP75 human pituitary tumor cell line identified by gene expression profiling. Endocrine. 33, 62-76.

58. Jin, L., Kulig, E., Qian, X., Scheithauer, B.W., Eberhardt, N.L., and R.V. Lloyd. 1998. A human pituitary adenoma cell line proliferates and maintains some differentiated functions following expression of SV40 large t-antigen. Endocrine Pathology. 9, 169-184.

APPENDIX

APPENDIX A

Conditional induction of ovulation in mice

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Conditional Induction of Ovulation in Mice[1]

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ABSTRACT

Follicle-stimulating hormone controls the maturation of mammalian ovarian follicles. In excess, it can increase ovulation (egg production). Reported here is a transgenic doxycycline-activated switch, tested in mice, that produced more FSHB subunit (therefore more FSH) and increased ovulation by the simple feeding of doxycycline (Dox). The transgenic switch was expressed selectively in pituitary gonadotropes and was designed to enhance normal expression of FSH when exposed to Dox, but to be regulated by all the hormones that normally control FSH production in vivo. Feeding maximally effective levels of Dox increased overall mRNA for FSHB and serum FSH by over half in males, and Dox treatment more than doubled the normal ovulation rate of female mice for up to 10 reproductive cycles. Lower levels of Dox increased the number of developing embryos by 30%. Ovarian structure and function appeared normal. In summary, gene switch technology and normal FSH regulation were combined to effectively enhance ovulation in mice. Theoretically, the same strategy can be used with any genetic switch to increase ovulation (or any highly conserved physiology) in any mammal.

assisted reproductive technology, anterior pituitary, follicle-stimulating hormone, gene regulation, ovulation

INTRODUCTION

Transgenes are difficult to express appropriately in vivo. Most often they have been controlled by promoters that possess little or no natural regulation over them, so they are expressed at happenstance levels throughout the animal's lifetime (embryo, neonate, adult) in many inappropriate tissues and without natural homeostatic control mechanisms to keep expression within normal physiological boundaries. A transgene for growth hormone, for instance, was used to accelerate growth and decrease body fat in pigs, but its unregulated high production caused a number of unwanted health problems [1]. A similar use of transgenes that expressed both subunits of FSH in many cells increased overall FSH expression in mice, but the increase overwhelmed and down-regulated the ovary causing infertility [2]. Because of problems associated with inappropriate expression of transgenes in vivo, an authoritative review on transgenic pigs concluded, "Regulatory sequence that will permit full control of gene expression must be developed before the full potential of gene transfer . . . can be realized" [1].

Gene switches like the one used in this report [3, 4] hold the promise of temporal control of transgenes so that their expression can be induced at the appropriate time in the embryo, neonate, or adult. Gene switches also act as rheostats that can gradually increase the level of transgene expression based on their level of activation. Nevertheless, gene switches are rarely used in vivo and are restricted almost entirely to applications involving cells in culture. A significant barrier to the use of transgenes in vivo to augment normal physiologies is the administration of gene switch activators at just the right times to mimic normal complex regulation, which is laboriously time-consuming and can never accurately duplicate normal regulation.

Presented here is a transgenic strategy that used a gene switch to effectively increase expression of FSH to reliably increase ovarian performance (increased ovulation rate) without the side effects associated with constitutive overexpression of FSH. This report details an achievement that can be used to increase understanding of germ cell production in females and males and also lead to increased reproductive efficiency in laboratory and farm animals. Of equal importance, the strategy can be used as a model for enhancing any highly conserved and important physiology that is central to the life of any eukaryote. Such genetic control can serve as a research tool to gain information about the basic regulation of important physiologies or it can be used in practice to control key physiologies to correct genetic defects, enhance health or improve phenotypic characteristics.

MATERIALS AND METHODS

Constructing Tetracycline-Sensitive Gene Switches

The *Tg(FSHB-rtTA, tetO-FSHB)* gene switch (Fig. 1) was made by cloning the promoter/intron (−4741 base pair [bp] to +759 bp) for the ovine FSHB subunit [5] into the tetracycline gene switch as it occurs in pKBMpMCR [6]. In this construct, the promoter for ovine FSHB controls expression of the reverse tetracycline-controlled transactivator, rtTA (see Fig. 1, left bold box). The promoter for FSHB is known to direct expression of genes selectively to pituitary gonadotropes in mice, where they are regulated just like the endogenous mouse gene for FSHB [7, 8]. The 5.5 kb *Pst*1 fragment of the ovine promoter/intron for FSHB was cloned into pKBMpMCR using *Not*1 and *Bgl*2 restriction sites added by pZero Blunt TOPO subcloning (Invitrogen Life Technologies, Carlsbad, CA). Then the structural gene for ovine FSHB (+1 bp to +3061 bp) [9] was cloned into the construct using polymerase chain reaction (PCR)-generated *Mlu*1 and *Pvu*1 sites. An analogous gene switch, *Tg(CGA-rtTA, tetO-FSHB)*, was made using the human alpha-glycoprotein subunit promoter (−315 bp to +45 bp) [10] to drive expression of rtTA specifically in pituitary gonadotropes in mice. For this cloning, *Not*1 and *Bgl*2 restriction sites were created as above. The pKBMpMCR parent construct contains kanamycin resistance for growth on bacterial agar plates as well as neomycin (or G418) resistance for the stable transformation of eukaryotic cells.

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Expressing Tg(CGA-rtTA, tetO-FSHB) *or* Tg(FSHB-rtTA, tetO-FSHB) *Gene Switches in LβT2 Cells*

LβT2 cells were cultured as reported previously [11] and transiently transfected with constructs containing either *Tg(CGA-rtTA, tetO-FSHB)* or *Tg(FSHB-rtTA, tetO-FSHB)* in 24-well tissue culture plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) using Lipofectamine Plus (Invitrogen, Carlsbad, CA) and 1 μg of DNA/well. For stable incorporation of transgenes into LβT2 cells, cultures were treated after transfection with 500 μg/ml of geneticin (G418; Sigma, St Louis, MO). Surviving colonies were expanded and tested for their ability to produce FSH when treated with or without 1 μM Dox for 48 h, and the media were assayed for FSH by radioimmunoassay (RIA; see below). For dose-response studies, cultures were treated for 48 h with 0.01, 0.1, 1 or 10 μM Dox and then culture media were assayed by RIA for FSH.

Universal RIA for FSH (Pan-FSH RIA)

FSH was measured using a pan-FSH RIA distributed by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Bethesda, MD) using a double antibody method [11]. All samples were assayed in duplicate from at least triplicate samples; the slope was −1.1 logit units per log unit of FSH, and intra-assay variation was ≤8%. Culture media and serum were stored at −20°C until analysis. Serum was obtained from clotted whole blood collected by heart puncture in killed mice. The RIA used rabbit anti-ovine FSH antiserum (AFP-C5288113) as first antibody, and purified mouse FSH (AFP-5308D) was used as tracer and reference protein. The NIDDK indicated that the RIA could not distinguish between FSH from different mammalian species, so it was considered a "universal" assay for FSH. The second antibody was sheep anti-rabbit antiserum prepared in our laboratory.

Generation and Screening of Transgenic Mice

All mouse experiments were approved by the Institutional Animal Care and Use Committee of North Carolina State University or the University of North Carolina at Chapel Hill. Constructs carrying *Tg(FSHB-rtTA, tetO-FSHB)* or *Tg(CGA-rtTA, tetO-FSHB)* were restricted with *Srf*1 and injected into zygotes from B6/SJL mice by the Animal Model Core Facility at the University of North Carolina, Chapel Hill, NC. Transgenic mice were identified by tail DNA as reported [7] using PCR oligonucleotides that detect sequences in the gene encoding rtTA of pKBMpMCR (435 bp to 1344 bp). The forward oligonucleotide was 5′-CAAGAGCTTC AGATGTGCCCTG-3′ and the reverse was 5′-GAATGCAATTGTTGTT GTTAAC-3′ (Sigma Genosys, The Woodlands, TX).

Measuring Mouse and Ovine mRNAs Encoding FSHB

Pituitaries were obtained within 5 min of death and homogenized in Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) to obtain total RNA according to Tri Reagent instructions. Total RNA was quantified by absorbance at 260 ηm, and 2 μg from each pituitary sample was converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Inc., Hercules, CA). Portions of iScript cDNA (equivalent to 100 ηg of original RNA) were assayed in triplicate by either PCR or real-time PCR. Oligomers for all PCR products were designed to detect mRNAs encoding FSHB of either mouse (endogenous) or ovine (switch-made) origin, and their products spanned intron II of both genes. Samples assayed without the iScript conversion showed no product formation.

Initial screening of transgenic mice for pituitary-specific expression and Dox-mediated induction of mRNA for ovine FSHB employed semiquantitative PCR with the following oligomer primers from Sigma Genosys: 5′-GACGTAGCTGTTTACTTCCCAG-3′ (forward) and 5′-CACAG CCAGGCAATCTTACGGTC-3′ (reverse) for mRNA encoding mouse FSHB and 5′-CAGGATGAAGTCCGTCCAG-3′ (forward) plus 5′-CTC TTTATTCTCTGATGTGACTGAAG-3′ (reverse) for mRNA encoding ovine FSHB. Fragment sizes obtained using these primers were 280 bp and 399 bp for mRNAs encoding mouse and ovine FSHB, respectively, as expected (data not shown).

Primers and probes for real-time reverse transcription-polymerase chain reaction (RT-PCR) were obtained from Integrated DNA Technologies, Inc, (Coralville, IA). Oligomers for mouse cDNA were 5′-AGAGA AGGAAGAGTGCCGTTTCTG-3′ (forward) and 5′-ACATACTTTCTG GGTATTGGGCCG-3′ (reverse), plus the Taqman probe (FAM) 5′-ATC AATACCACTTGGTGTGCGGGCTA-3′. Oligomers for ovine cDNA were 5′-ACCGTGGAGAAAGAGGAATGTAGC-3′ (forward) and 5′-TA-CATGCTTTCTGGATGTTGGGCCTT-3′ (reverse) plus the Taqman probe (FAM) 5′TGCATAAGCATCAACACCACGTGGTG-3′. Real-time PCR was performed in the iCycler (Bio-Rad) according to the manufacturer's instructions.

Real-time RT-PCR was specific for the mRNAs encoding either mouse or ovine FSHB because of sequence differences. Absolute quantitation of these mRNAs was performed by comparing "ct" values of samples and standards; standards were made by producing cDNAs from total RNA extracts using the iScript procedure followed by cloning into pZero Blunt TOPO. The standard constructs were quantified using Hoefer Dyna Quant 200 fluorometer (Amersham, Pharmacia Biotech) and numbers of RNA molecules were calculated by comparing ct values of unknowns to ct values of either mouse or ovine standards. The standard assay lines for both mouse and ovine cDNA clones were superimposable with identical slopes and potencies. Variation of unknown values was ≤10% within or between assays.

Ovulation

Females were exposed to bedding from males for 1 wk before feeding Dox and were fed rodent chow with or without Dox (6 g/kg rodent chow; Bioserv, Frenchtown, NJ) starting 2 or 30 days before being placed with fertile males (one male per two females). Females were checked each morning at 0900 h for copulation plugs, the presence of which suggested that ovulation occurred during the previous 9 h. By 1200 h each day, females with plugs were killed, and were analyzed for ovulation by quantifying eggs in both ampullae. Rarely, ovulation did not occur or was extremely low, perhaps reflecting activity from only one ovary. This occurred equally for control and Dox-fed mice, so these data were not included.

Some mice were fed Dox at 0.2 g/kg rodent chow, which is the standard food routinely sold by Bioserv for inducing doxycycline-sensitive transgenes in vivo. These mice were treated just like those fed the higher-Dox diet (6 g/kg rodent chow). Ten days after the copulation plug appeared, mice were killed and the developing embryos were counted. In all cases, the embryos were ~5 mm in diameter, and all appeared healthy based on inspection through the uterine wall. In a follow-up experiment, mice were fed the high-Dox diet (6 g Dox/kg rodent chow) and pregnancies were carried to term. All mice were born vigorous and healthy with no apparent defects.

Analyzing Ovarian Follicles

The ovaries of mice treated with or without Dox were fixed in Bouin solution for ≥24 h, sectioned in wax, and stained with hemotoxylin and eosin under standard conditions. Each ovary was sectioned from beginning to end in groupings of four sections of 5 μm thickness each. These groupings were interspersed with 50 μm of tissue that was not sectioned. To avoid double counting of tertiary follicles that averaged 200 μm in diameter, every third grouping of four sections was analyzed carefully. Primary, secondary and tertiary follicles were defined as described previously [12] and counted. Care was taken not to double-count follicles. Tertiary follicles were counted and measured with an eyepiece micrometer in sections only where the cut bisected the developing oocyte. Primordial follicles were not analyzed because FSH has never been shown to significantly affect them. The data represent the mean ± SEM from females fed rodent chow with or without Dox for 30–39 days (six treated, six controls). Mice fed rodent chow with or without Dox for 2–11 days (five treated, five controls) were also analyzed with identical results (data not shown).

Statistics

Statistical calculations were performed using software from Prism 4 (GraphPad Software, Inc., San Diego, CA) and SAS (SAS Institute, Inc, Cary, NC). To determine differences between several means, analysis of variance (ANOVA) was used followed by a Tukey multiple comparison test for post hoc evaluation of differences between different treatment groups. Differences between two means were analyzed using a Student *t*-test. Differences between the slopes of lines (FSH RIA and real-time PCR analyses) were analyzed using the PROC REG procedure of SAS. All experiments were performed with triplicate replicates, with individual samples being assayed in duplicate. Means ± SEM are plotted in all figures except Figure 4, in which individual data points are displayed.

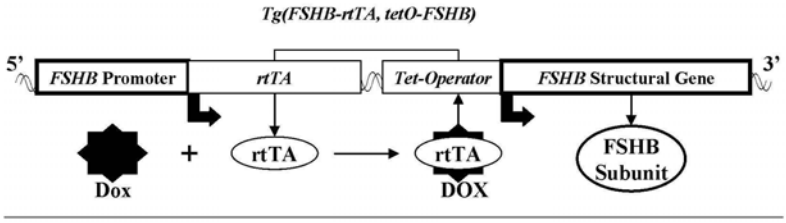

FIG. 1. The ovine *Tg(FSHB-rtTA, tetO-FSHB)* gene switch. The promoter for ovine FSHB (left box) was used to control expression of the reverse tetracycline-sensitive transactivator (rtTA). This promoter has been shown to express other genes specifically in gonadotropes where they are regulated just like endogenous mouse FSHB subunit protein. Activation of rtTA by doxycycline (Dox), a tetracycline analog, was designed to induce expression of the structural gene for ovine FSHB (right box) by binding to the *Tet* operator DNA sequence. Because expression of rtTA is designed to mimic expression of endogenous mouse FSHB, Dox activation of rtTA should simply amplify normal regulation of FSHB expression leading to enhanced production of FSHB and FSH in a way that reflects the normal ebb and flow of endogenous FSH.

RESULTS

Testing FSH Production in LβT2 Cells Using Two Gene Switches

Figure 1 is a diagram of the *Tg(FSHB-rtTA, tetO-FSHB)* gene switch that functioned successfully in our studies to increase FSH and ovulation in transgenic mice. Expression of this switch was controlled by the promoter for ovine FSHB, a promoter known to target expression to pituitary gonadotropes [7, 8]. An analogous gene switch, *Tg(CGA-rtTA, tetO-FSHB)*, was controlled by the promoter for the human alpha-glycoprotein subunit that is also known to target gene expression to gonadotropes [10]. Both gene switches were produced as described in *Materials and Methods*, and were shown to function well in LβT2 cells (Fig. 2).

Figure 2 shows Dox-induced expression of FSH in individual LβT2 colonies stably expressing either *Tg(CGA-rtTA, tetO-FSHB)* or *Tg(FSHB-rtTA, tetO-FSHB)*. The LβT2 cells naturally express mouse alpha-glycoprotein subunit [13], but cannot produce measurable amounts of FSHB or FSH without activin treatment [13, 14]. Basal expression and induction of each transgene were measured by the ability of each colony to produce FSH when treated with Dox; FSH was quantitatively measured by the pan-FSH RIA. Maximal levels of Dox (10 μM) induced FSH production 3- to 20-fold with *Tg(FSHB-rtTA, tetO-FSHB)* and 4- to 100-fold with *Tg(CGA-rtTA, tetO-FSHB)* as shown in Figure 2. Dose-response data obtained with three colonies expressing *Tg(CGA-rtTA, tetO-FSHB)* showed that the ED_{50} for Dox was 0.3–1.0 μM, as expected [3, 4, 6]. These data proved that both of the FSHB-producing gene switches functioned as intended and that the endogenous mouse alpha-glycoprotein subunit effectively joined with ovine FSHB (Dox-induced FSHB) to produce a chimeric mouse/ovine molecule of FSH.

Screening transgenic mice for FSH and mRNA encoding FSHB

Ten founder mice harboring the *Tg(FSHB-rtTA, tetO-FSHB)* gene switch [*Tg(FSHB-rtTA, tetO-FSHB)1–10Wmil*] and fourteen founders carrying the *Tg(CGA-rtTA, tetO-FSHB)* gene switch [*Tg(CGA-rtTA, tetO-FSHB)1–14Wmil*] were produced and mated with CD-1 mice. When the offspring were reproductively mature (>7 wk old), males were treated ± Dox for 2 wk and then killed, and blood was assayed for serum FSH by RIA; mRNA encoding mouse or ovine FSHB was quantified using semiquantitative PCR. Males were used to avoid the wide variation found in female FSH values, which is presumably caused by differences in reproductive cycle stage that are difficult to assess.

Initial RT-PCR screening found that founder lines *Tg(FSHB-rtTA, tetO-FSHB)1–6Wmil* expressed mRNA for FSHB specifically in the pituitary (luciferase activity [RLU/mg protein] was 99 times higher in pituitary than in testis or liver) and Dox increased mRNA for FSHB 2- to 4-fold. Likewise, founder lines *Tg(CGA-rtTA, tetO-FSHB)1–5Wmil* showed preferential expression in pituitary tissue. In all cases, Dox increased levels of mRNA for FSHB in pituitary tissue and also appeared to increase overall FSH in mouse serum, but increases in serum FSH were not statistically significant because of high variation between mice, both male and female. Based on these initial RT-PCR data, two founder lines, *Tg(FSHB-rtTA, tetO-FSHB)1&2Wmil*, were expanded to determine if Dox treatment in vivo would increase ovulation rate in females. Measuring mRNA for FSHB by real-time RT-PCR was deemed the best way to determine the presence of a functional transgene, and real-time RT-PCR was used to accurately quantify mRNAs for endogenous and/or ovine FSHB subunits throughout this report.

Real-time RT-PCR was first used to accurately quantify tissue-specific expression of ovine *FSHB* mRNA in *Tg(FSHB-rtTA, tetO-FSHB)1Wmil*, which was the mouse line ultimately chosen for study in this report (see superovulation below and Fig. 3). The data in Figure 3 indicate that fully induced male expression of *FSHB* mRNA (2 wk of Dox at 6 g/kg rodent chow) was highly specific for pituitary gonadotropes because expression was <2% in forebrain, heart, lung, liver, spleen, and gonads compared to pituitary expression.

Superovulation by Dox

The ability of transgenes to increase ovulation was tested on 12 females each from three separate founder mouse lines: *Tg(FSHB-rtTA, tetO-FSHB)1&2Wmil* and *Tg(CGA-rtTA, tetO-FSHB)1Wmil*. Treatment of *Tg(FSHB-rtTA, tetO-FSHB)1&2Wmil* females with Dox (6 g/kg of rodent chow) equally increased ovulation in both founder lines from 12 oocytes up to 20–40 (or 90 in one case) per ovulatory cycle. The one founder line chosen from mice containing the *Tg(CGA-rtTA, tetO-FSHB)* gene switch did not exhibit increased ovulation when fed Dox (data not shown). Because both *Tg(FSHB-rtTA, tetO-FSHB)1&2Wmil* gave similar Dox-mediated increases in ovulation, only one line, *Tg(FSHB-rtTA, tetO-FSHB)1Wmil*, was expanded to produce mice for larger scale studies.

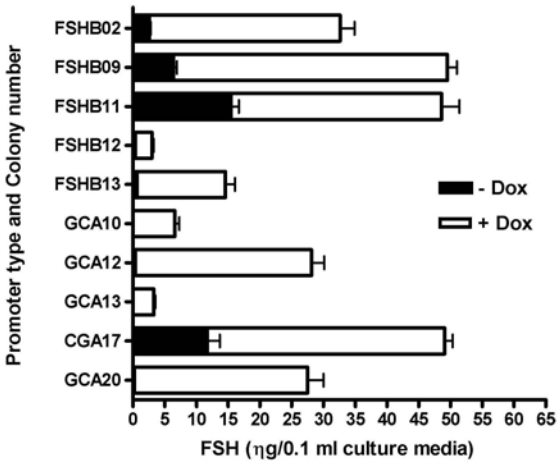

FIG. 2. Induction of FSH expression by Dox in LβT2 cells harboring the *Tg(CGA-rtTA, tetO-FSHB)* or *Tg(FSHB-rtTA, tetO-FSHB)* gene switches. Five cell lines stably carrying the *Tg(CGA-rtTA, tetO-FSHB)* gene switch and five cell lines expressing *Tg(FSHB-rtTA, tetO-FSHB)* were cultured for 48 h with or without 10 μM Dox. The media were assayed by a pan-FSH RIA and the means ± SEMs are shown for triplicate replicates assayed in duplicate for all cell lines. Expression of FSH was significantly increased above control levels for all cell lines ($P < 0.001$).

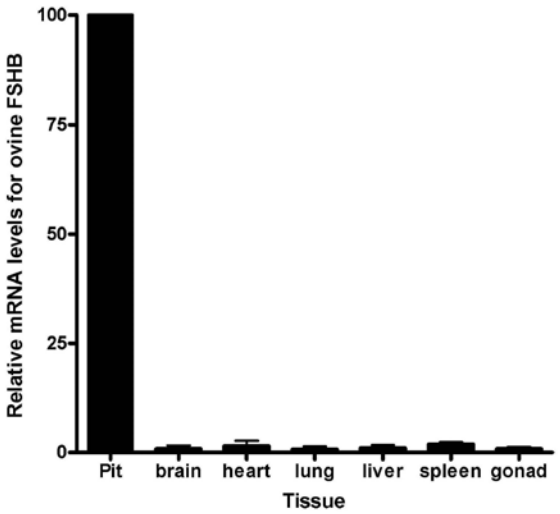

FIG. 3. Pituitary-specific expression of mRNA for ovine FSHB in *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* mice during Dox treatment. Male mice from *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* founders were treated with Dox for two weeks, and then tissues were taken and assayed for mRNA of ovine FSHB using real-time RT-PCR. All samples from individual mice were assayed together and the results were normalized to pituitary expression of "100." To determine differences between several means, analysis of variance was used, followed by the Tukey multiple comparison test for post hoc evaluation of differences between different treatment groups. Assay variation was ≤10%. Individual percentage expressions for brain, heart, lung, liver, spleen, and gonad were 0.9 ± 0.7, 1.5 ± 1.2, 0.7 ± 0.7, 1.0 ± 0.7, 1.9 ± 0.6, and 0.9 ± 0.4, respectively, compared to expression of mRNA for FSHB in the pituitary.

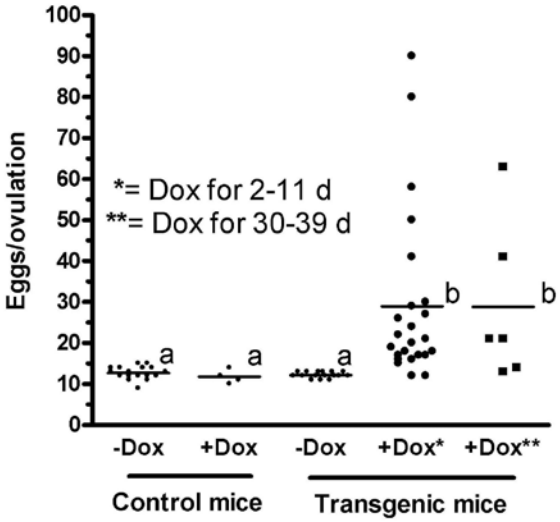

FIG. 4. *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* females increased ovulation rates by 240% ± 33% when fed Dox (6g/kg rodent chow) for 2–11 or 30–39 days. Three-month-old hemizygous mice were treated with Dox for 2 or 30 days and then exposed to males. All females showed evidence of copulation (copulation plug) suggesting ovulation within 9 days of exposure to males; >80% showed a plug within 4 days of male exposure. Homozygous mice were also tested at 3 mo of age and showed an ovulation rate of 29 ± 4. Moreover, hemizygous and homozygous mice were tested at age 7 mo with the same increase in ovulation for Dox-fed mice compared to controls. Means of treatment values are designated with a line horizontal to the x-axis. Means that are statistically the same ($P < 0.05$) have the same letter (a or b).

Figure 4 shows that females from *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* increased ovulation by 240% ± 33% when fed Dox (6 g/kg rodent chow). Control CD-1 mice ovulated 12 ± 0.5 eggs with or without Dox treatment, as did transgenic female mice without Dox. With Dox, *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* females produced 29 ± 4 eggs per ovulatory cycle. Moreover, it made no difference whether mice were 3 or 7 mo old (only data from 3 mo old mice are shown) or if the transgene was hemizygous or homozygous (only data from hemizygous mice are shown), and it made no difference if Dox was given for 2–11 days (1–3 estrous cycles) or 30–39 days (7–10 estrous cycles). These data show that exposure to Dox for up to 10 reproductive cycles increased ovulation an average of 240%, presumably at every ovulation (Fig. 4).

To investigate the viability of ovulated oocytes, transgenic hemizygous mice were treated as in Fig. 4 (see 2–11-day results), but with a lower dose (0.2 g/kg rodent chow) to avoid producing the 30–50 ovulations associated with feeding the higher level of Dox diet (6 g/kg rodent chow). The extremely high ovulation rates often resulted in uterine overcrowding, multiple embryo absorption, and no overall gain in birth rate (data not shown). This study used 38 *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* females that were fed normal rodent chow (12 mice) or rodent chow containing Dox (26 mice) for 2 days before and during exposure to fertile males. Females were given normal rodent chow when plugs were observed, and mice were killed 10 days afterwards. Although embryos were not analyzed in detail, all embryos appeared uniform in size ~5 mm in diameter and looked as normal as the control embryos; there was no

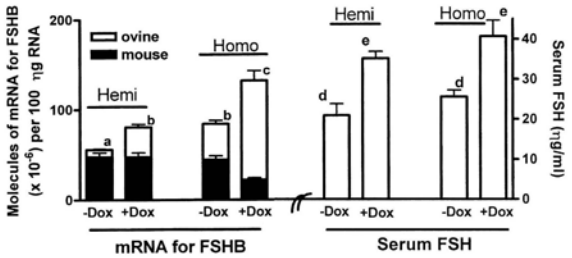

FIG. 5. Dox increased total FSH and mRNA for FSHB by 50%–60% in male *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* mice. No increase was observed in males lacking the fertility switch (not shown). Transgenic B6SJL founder mice were bred into CD-1 mice, giving them considerable CD-1 genetic makeup. Data from the fourth generation are presented in this report. Males were fed rodent chow ± 6g/kg of Dox for 14 days, and then their pituitaries and blood were analyzed. Total RNA was isolated, and mRNAs for ovine FSHB and mouse FSHB were quantified in 100 ηg of RNA using real-time RT-PCR. Standard curves for PCR analyses consisted of known amounts of cDNA for ovine or mouse FSHB in plasmids. Each data point represents the mean ± SEM for either four hemizygous or seven homozygous mice. Statistical analysis used analysis of variance plus the Tukey multiple comparison test. There are significant differences ($P < 0.05$) between means with different letters.

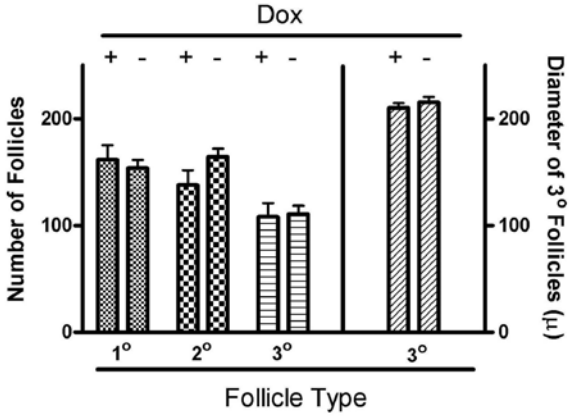

FIG. 6. Treatment with Dox for 30–39 days did not alter the number of primary, secondary or tertiary follicles or the size of tertiary follicles (Student *t*-test; $P < 0.05$). The data represent the means ± SEM for results from six control and six Dox-treated transgenic mice. The diameter of tertiary follicles is reported in microns.

evidence of embryo absorption in any of the Dox-fed mice. Without feeding Dox there was no statistical difference between embryo number in transgenic mice (12.7 ± 6) and normal CD-1 birthing rate (11.5 ± 0.2; n = 41) or CD-1 ovulation rate (see Fig. 4). Females fed Dox carried 30% more embryos (16.5 ± 0.8) than control CD-1 mice fed Dox. To test possible deleterious effects of extremely high levels of Dox, *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* females were fed the highest level of Dox (6 g/kg rodent chow) 2 days before mating and throughout the entire pregnancy. Birthing rate was 10.7 ± 1 per litter (not different from control mice, but unexpectedly low, presumably because of overcrowding of uterine space), and all offspring were vigorous and seemingly normal up through weaning, when the experiment was terminated.

Production of FSH and mRNA for FSHB in Tg(FSHB-rtTA, tetO-FSHB)1Wmil

Males were used for regulatory studies because cycle variation in females made these studies very difficult. Figure 5 shows changes in pituitary mRNA levels for mouse FSHB, ovine FSHB, and serum FSH in hemizygous or homozygous *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* males following treatment with Dox. Hemizygous or homozygous *Tg(FSHB-rtTA, tetO-FSHB)1Wmil* males were analyzed by real-time RT-PCR for endogenous mouse and switch-made mRNA for ovine FSHB. Total mRNA for mouse and ovine FSHB was plotted by stacking the bar for switch-made ovine *FSHB* mRNA (white) on top of the bar representing endogenous mouse *Fshb* mRNA (black). The data show that Dox increased total mRNA for FSHB by ~50% and serum FSH by 56% ± 5% over untreated mice (Fig. 5; Hemi or Homo). When homozygous males were used, Dox also increased total mRNA for FSHB and serum FSH by ~50% (see Homo), but endogenous mouse *Fshb* mRNA decreased by ~50% showing the effects of feedback inhibition.

Basal expression of mRNA for ovine FSHB occurred even without Dox treatment in both hemi and homozygous mice. Basal expression was 4-fold greater in homozygous mice so that the basal mRNA level for ovine FSHB actually equaled the fully stimulated level of mRNA for ovine FSHB in Dox-treated hemizygous mice (compare hemi+Dox with homo-Dox).

Ovarian Morphology With or Without Dox Treatment

The ovaries for this study were taken from the same mice analyzed for ovulation in Figure 4 that had been treated with or without Dox for 30–39 days. Data in Figure 6 show that there was no evidence for changes in the numbers of primary, secondary, or tertiary follicles, nor was there any difference in the average size of tertiary follicles. Furthermore, careful inspection of the ovaries showed no evidence of damage to the ovaries such as cyst formation, obvious depletion of primordial follicles, or any malformation in transgenic animals that were fed Dox over a 30–39 day period. In fact, the ovaries of mice fed Dox for 30–39 days were not different from those of control mice (see Fig. 6) except they contained more corpora lutea (corpora lutea were not quantified).

DISCUSSION

Reported here is a method for increasing ovulation rate using a doxycycline-sensitive gene switch to enhance production of FSH. There have been significant effort and resources expended in the past trying to achieve increased fertility in laboratory mice [15] and farm animals [16] by breeding for enhanced ovulation rate and/or uterine capacity. These traits segregate separately in complex ways that have permitted only limited success for increasing either the number of eggs ovulated or uterine capacity. The approach used here permitted reliable increases in ovulation that can be theoretically adjusted to any level between maximum enhancement and zero effect. Such a model system is likely to be useful for those studying oocyte maturation, ovulation and spermatogenesis or trying to breed for increased fertility, which may require increased uterine capacity.

The transgenic method used here differed significantly from others used in the past. This method was designed to use a gene switch as a simple amplifier of normal FSH

expression so that all the normal homeostatic complexity would be preserved (regulation by activins, inhibins, follistatin, estradiol, progesterone, GnRH, and possibly others) [17, 18]. Preferential expression of the transgene (>98% in the pituitary; Fig. 3) demonstrated that gonadotrope-specific expression was achieved just as with two other transgenes driven by the promoter for ovine FSHB [7, 8]. Considering that gonadotropes comprise <5% of mouse pituitary cells [8], the specificity of ovine FSHB expression in gonadotropes was calculated to be more than 1000 times greater compared to the other cell types tested. Furthermore, gonadotrope-specific regulation of *Tg(FSHB-rtTA, tetO-FSHB)* was demonstrated by the feedback inhibition on mRNA for endogenous mouse FSHB in homozygous mice (see Fig. 5). Therefore, the strategy used in this transgenic switch seems to have achieved significant exogenous control (induction by Dox treatment) while preserving physiological regulation of FSHB produced by the transgene. This was the goal articulated in the introduction of this paper as the prerequisite for achieving the full potential of modern transgenic technology [1].

The transgenic strategy used here stands in contrast to tetracycline-activated switches that are expressed constitutively at high levels using strong viral or eukaryotic promoters. In these more traditional constructs, regulation is controlled entirely by Dox. In fact, use of the strong alpha-glycoprotein subunit promoter tested in *Tg(CGA-rtTA, tetO-FSHB)1Wmil* may have failed because it was too much like a constitutively active viral promoter. Based on PCR data, mRNA for ovine FSHB was expressed and induced in the pituitaries of these mice, but it failed to increase ovulation, perhaps because it lacked regulation by an authentic promoter for FSHB. Because only one transgenic line was tested, however, it is impossible to know what caused the failure, because failure could have resulted from a number of factors, including location of the transgene in chromatin.

Mice that expressed *Tg(FSHB-rtTA, tetO-FSHB)* showed an increased ovulation rate of 240% whether they were analyzed 2 or 39 days after eating Dox. In fact, acute treatment with Dox (2 days) caused some mice to ovulate 40–50 oocytes. This was as effective as acute treatments with pregnant mare's serum gonadotropins and human chorionic gonadotropin [19]. Superovulation after this short time suggested that Dox rapidly increased FSH in mice that were in proestrus and almost ready to ovulate, which was apparently enough to rescue a significant number of mature follicles from apoptosis to more than double the ovulation rate. It was gratifying that superovulation occurred even after 39 days of continuous Dox treatment. These results suggested that expression of transgenic ovine FSHB mimicked the normal rhythm of endogenous mouse FSHB, enhancing it at just the right time before each ovulation of each estrous cycle. A related observation that reinforces the notion of enhanced but normal transgene regulation is the fact that 70–80% of mice ovulated within 4 days of exposure to males whether or not they were pretreated with Dox for 0, 2, or 30 days. This indicated that Dox had no effect on estrous cycle patterns in the mice, a fact that is consistent with transgenic and endogenous patterns of FSHB being similar.

It was of interest to determine whether expression and Dox activation of *Tg(FSHB-rtTA, tetO-FSHB)* had an effect on the number and/or nature of primary, secondary or tertiary follicles, because it is known that the latter are affected by FSH [20]. The data in Figure 6 indicate that Dox had no effect on the qualitative nature of any of the three types of follicles even after 30–39 days of treatment, suggesting that the transgene must have restricted its action primarily to rescuing follicles at the last minute before ovulation.

It is worth considering how the transgenic mice adjusted to the higher than normal levels of mRNA for FSHB produced by the "leaky" *Tg(FSHB-rtTA, tetO-FSHB)* gene switch even in the absence of Dox (Fig. 5). Presumably, this adjustment occurred as they developed in utero or neonatally. The data show that without Dox, pituitaries of hemizygous or homozygous males naturally produced ~10% or ~50%, respectively, more total mRNA for FSHB than pituitaries in normal CD-1 mice (Fig. 5). However, these transgenic mice did not have higher serum FSH or higher ovulation rates than normal CD-1 mice unless they were treated with Dox (Fig. 4). These data indicate that developmental mechanisms at the pituitary, and/or possibly the ovary, kept FSH levels and ovulation within boundaries associated with normal CD-1 mouse physiology [19]. These mechanisms are not presently understood but are clearly present.

Finally, the strategy used in this study to increase fertility should be useful for enhancing other physiologies in mammals. Growth hormone (GH), for example, shows peak secretion during the night in males [21] or throughout the day in females [22]. If a GH gene switch were designed like the *Tg(FSHB-rtTA, tetO-FSHB)* gene switch, using the GH locus control region and regulatory sequences in its proximal promoter [23], an appropriate amount of Dox (or nonantibiotic tetracycline derivative [24, 25]) could be administered during high growth periods to increase lean protein deposition in farm animals in a normal way that should not interfere with other physiologies. With reference to human medicine, the ability to control genetic diseases with genes expressed in somatic cells has made significant progress. It may be possible to control this type of gene therapy precisely using a gene switch arrangement like that of the *Tg(FSHB-rtTA, tetO-FSHB)* switch described here. An insulin gene switch, for example, could be used as a transgene targeted specifically to insulin-producing pancreatic cells [26], where it could be regulated by Dox along with its own cell-specific homeostatic control mechanisms. Likewise, any peptide hormone could be enhanced in a regulated way in other mammals (or plants).

In summary, a method has been devised to use a ligand-activated gene switch to enhance production of a hormone (in this case FSH) in a physiologically effective way in mammals. This approach has been seldom used for expressing useful transgenes in mammals, and therefore represents a useful strategic shift for regulating transgenes in vivo. The method has immediate value for those studying oocyte maturation, ovulation, uterine capacity, and spermatogenesis. It has potential application for increasing ovulation and birthing rates not only in mice, but in larger mammals as well. Finally, it may have broader application to the regulation of any transgene in any organism including humans.

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transgenes. Dr. John Nilson (Pullman, WA) is thanked for his generous gift of the promoter for human CGA.

REFERENCES

1. Pursel VG, Bolt DJ, Miller KF, Pinkert CA, Hammer RE, Palmiter RD, Brinster RL. Expression and performance in transgenic pigs. J Reprod Fertil Suppl 1990; 40:235–245.
2. Kumar TR, Palapattu G, Wang P, Woodruff TK, Boime I, Byrne MC, Matzuk MM. Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. Mol Endocrinol 1999; 13:851–865.
3. Zhu Z, Sheng T, Lee CG, Homer RJ, Elias JA. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin Cell Dev Bio 2002; 13:121–128.
4. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995; 268:1766–1769.
5. Miller CD, Miller WL. Transcriptional repression of the ovine follicle-stimulating hormone-beta gene by 17 beta-estradiol. Endocrinology 1996; 137:3437–3446.
6. Wells KD, Foster JA, Moore K, Pursel VG, Wall RJ. Codon optimization, genetic insulation, and an rtTA reporter improve performance of the tetracycline switch. Transgenic Res 1999; 8:371–381.
7. Huang H-J, Sebastian J, Strahl BD, Wu JC, Miller WL. The promoter for the ovine follicle-stimulating hormone-β gene (FSHβ) confers FSHβ-like expression on luciferase in transgenic mice: regulatory studies in vivo and in vitro. Endocrinology 2001; 142:2260–2266.
8. Wu JC, Su P, Safwat NW, Sebastian J, Miller WL. Rapid, efficient isolation of murine gonadotropes and their use in revealing control of follicle-stimulating hormone by paracrine pituitary factors. Endocrinology 2004; 145:5832–5839.
9. Guzman K, Miller CD, Phillips CL, Miller WL. The gene encoding ovine follicle stimulating hormone β: isolation, characterization, and comparison to a related ovine genomic sequence. DNA Cell Biol 1991; 10:593–601.
10. Jorgensen JS, Quirk CC, Nilson JH. Multiple and overlapping combinatorial codes orchestrate hormone responsiveness and dictate cell-specific expression of the genes encoding luteinizing hormone. Endocr Rev 2004; 25:521–542.
11. Huang HJ, Wu JC, Su P, Zhirnov O, Miller WL. A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology 2001; 142:2275–2283.
12. Rugh R. The Mouse. Minneapolis, MN: Burgess Publishing Co; 1968: 31.
13. Graham KE, Nusser KD, Low MJ. LbetaT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to activin A. J Endocrinol 1999; 162:R1–R5.
14. Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW. Cell-specific transcriptional regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell model. Endocrinology 2001; 142: 2284–2295.
15. Clutter AC, Kirby YL, Nielsen MK. Uterine capacity and ovulation rate in mice selected 21 generations on alternative criteria to increase litter size. J Anim Sci 1994; 72:577–583.
16. Ford JJ, Zimmerman DR, Wise TH, Leymaster KA, Christenson RK. Increased plasma follicle-stimulating hormone concentrations in prepubertal gilts from lines selected for increased number of corpora lutea. J Anim Sci 2001; 79:1877–1882.
17. Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW. Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Mol Cell Endocrinol 2004; 225:29–36.
18. Winters SJ, Moore JP. Intra-pituitary regulation of gonadotrophs in male rodents and primates. Reproduction 2004; 128:13–23.
19. D'Cruz OJ, Uckun FM. Lack of adverse effects on fertility of female CD-1 mice exposed to repetitive intravaginal gel-microemulsion formulation of a dual-function anti-HIV agent: aryl phosphate derivative of bromo-methoxy-zidovudine (compound WHI-07). J Appl Toxicol 2001; 21:317–322.
20. McGee EA, Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 2000; 21:200–214.
21. Dimaraki EV, Jaffe CA, Bowers CY, Marbach P, Barkan AL. Pulsatile and nocturnal growth hormone secretions in men do not require periodic declines of somatostatin. A. J Physiol Endocrinol Metab 2003; 285:E163–E170.
22. Klerman EB, Adler GK, Jin M, Maliszewski AM, Brown EN. A statistical model of diurnal variation in human growth hormone. Am J Physiol Endocrinol Metab 2003; 285:E1118–1126.
23. Jin Y, Surabhi RM, Fresnoza A, Lytras A, Cattini PA. A role for A/T-rich and Pit-1/GHF-1 in a distal enhancer located in the human growth hormone locus control region with preferential pituitary activity in culture and transgenic mice. Mol Endocrinol 1999; 13:1249–1266.
24. Greenwald R, Golub L. Biologic properties of non-antibiotic, chemically modified tetracyclines (CMTs): a structured, annotated bibliography. Curr Med Chem 2001; 8:237–242.
25. Chrast-Balz J, Hooft Van Huijsduijnen R. Bi-directional switching with the tetracycline repressor and novel tetracycline antagonist. Nucleic Acids Res 2004; 24:2900–2904.
26. Sasaki T, Fujimoto K, Sakai K, Nemoto M, Nakai N, Tajima N. Gene and cell based therapy for diabetes mellitus: endocrine gene therapeutics. Endocr Pathol 2003; 14:141–144.