ABSTRACT

SAFWAT, NEDAL WAFIK. Activin Induction of Follicle Stimulating Hormone is Mediated by Transforming Growth Factor Beta Activated Kinase-1 (TAK-1) in Pituitary Gonadotropes (Under the direction of William L. Miller.)

Follicle stimulating hormone (FSH) is an essential hormone for female folliculogenesis and plays an important role in male spermatogenesis. The hormone is secreted by pituitary gonadotropes in the anterior pituitary lobe, and its overall production is regulated by expression of the FSHβ subunit. The regulation of FSHβ subunit is achieved by combined actions of neurocrine, endocrine, and pituitary paracrine/autocrine factors. Activin shown to be produced locally within the pituitary is a potent stimulator of the FSHβ subunit.

This study used 4.7 kb of the ovine FSHβ promoter linked to luciferase (oFSHβLuc) plus a well characterized activins responsive construct, p3TPLuc, to investigate the hypothesis that Smad3, TAK1 (TGFβ activated kinase1), or both cause activin-mediated induction of FSH. Over-expression 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 > 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-mitogen activated protein kinase (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.

In addition, a method was developed in our laboratory for purifying primary gonadotropes to study the solitary role of factors regulating FSHβ gene. We were able to isolate primary gonadotropes from pituitary with purities higher than 95%. The data show that gonadotropes are able to produce activin and/or activin-like molecule(s), however paracrine factors from pituitary non-gonadotropes play a major role in controlling FSHβ at the pituitary level. Overall, the study presented provides an understanding to the TAK1 signaling pathway mediating activin induction of FSHβ, and shows that primary gonadotropes rely on paracrine factors to produce activin.

Activin Induction of Follicle Stimulating Hormone is Mediated by Transforming Growth Factor Beta Activated Kinase-1 (TAK-1) in Pituitary Gonadotropes

by

Nedal W. Safwat

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Approved by:

William L. Miller (Chair)	Dennis T. Brown
Carla Mattos	David W. Schomberg

DEDICATION

To my parents: *Wafik and Nagwa Safwat*

To the love of my life, my friend, my wife: *Miral Hassan*

BIOGRAPHY

Nedal Safwat was raised in Cairo, Egypt by his father Wafik Safwat and his mother Nagwa El-Gindy. He moved on his own to USA for a better education where he lived with his aunt Mimi Moghazi to attend the last 2 years of high school at Apex High School graduating in 1993 with honors. Nedal attended North Carolina State University where he graduated Summa Cum Laude with a Bachelor's Degree honors in Biochemistry. Nedal then worked at Bayer Pharmaceuticals for one year. Having interest in becoming a scientist, Nedal moved on to pursue scientific research. He first joined the department of biochemistry at Wake Forest University. After one year, Nedal transferred to department of biochemistry at North Carolina State University, where he joined Dr. William L. Miller lab to continue his graduate research. Nedal is currently working for Miltenyi Biotec Inc. pursuing a career in industry as he awaits the completion of his doctoral degree.

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Literature Review

The mammalian reproductive axis is regulated primarily by hormones from the hypothalamus, pituitary gland, and gonads. The hypothalamus contains neurosecretory cells that produce gonadotropin releasing hormone (GnRH) which stimulates endocrine cells in the anterior pituitary named gonadotropes to synthesize and release follicle stimulating hormone (FSH) and luteinizing hormone (LH). In males, FSH secreted from the pituitary plus testosterone help initiate spermatogenesis (sperm production). In females, FSH and LH initiate the development of the follicles (folliculogenesis) within the ovaries. The change in levels of GnRH, FSH, LH, and gonadal hormones (estrogen, testosterone, and progesterone) dictates the progression of the menstrual cycle (1).

FSH is essential for female fertility as shown in knockout studies, where FSH knockout mice are infertile due to early arrest in folliculogenesis (2). These studies relate to a condition in human females named hypergonadotropic dysgenesis (ODG), where loss of fertility is observed due to inactivating point mutation in the FSH receptor on the gonads (3). In males, FSH knock out mice are still fertile showing reduced testes size and low sperm count, similarly human male patients with ODG also show the same phenotypes (2, 3).

FSH belongs to the family of pituitary glycoprotein hormones (GPH) (4). The GPH family of proteins are heterodimers formed by association of a common α-glycoprotein subunit (α-GSU, 92 amino acids) and a unique β-Subunit that dictates the mature

hormone (5). Synthesis of the FSHβ subunit is rate limiting to the production of the biologically active hormone, so regulation of the FSHβ gene has been the focus for studying the overall production of FSH hormone [Figure 1; (4)].

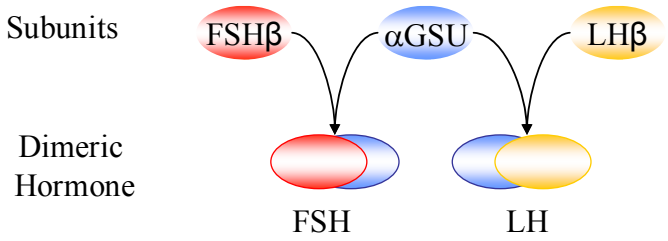


Figure 1: Subunit assembly of FSH and LH mature hormones.
Mature FSH and LH hormones are formed by assembly of common α-GSU subunit and FSHβ subunit to form FSH or LHβ subunit to form LH.

1. FSH Regulation by Hypothalamic and Pituitary Autocrine/Paracrine Factors:

The hypothalamus secretes GnRH that is delivered to pituitary gonadotropes to stimulate the production of FSH and LH. Secretion of GnRH from the hypothalamus occurs in a pulsatile manner, where the pulse frequency differentially regulates FSH and LH production. Slow frequency and low amplitude GnRH pulses stimulate FSHβ expression, whereas rapid pulse stimulates LHβ (4, 6-9).

It was later shown that FSH secretion is not entirely dependent on GnRH from the hypothalamus, but is also regulated by local factors within the pituitary gland (10-14). These local factors are secreted and through an autocrine/paracrine mechanism are able to stimulate FSH production. So far studies have identified inhibins, follistatin (FS),

activins, and bone morphogenetic proteins (BMPs) as the key paracrine/autocrine factors that regulate FSH expression within the pituitary [Figure 2; (10-15)].

1A. Evidence for non-Hypothalamic Factors Regulating FSH

The presence of autocrine/paracrine control within the pituitary was first demonstrated by Bishop et al. The study showed that lesions in the median eminence (a region that links the hypothalamus to the pituitary gland) of rats did not alter basal serum FSH levels, while LH levels were reduced (16). Later studies also showed that the administration of high doses of bioneutralizing anti-GnRH antibodies or GnRH antagonist did not alter basal FSH levels, while LH levels were suppressed by 50% (17, 18). The in vivo studies clearly demonstrated that LH expression is dependent on GnRH, while FSH is still expressed in the absence of GnRH through non-hypothalamic factors.

1B. Identification of Pituitary Paracrine/Autocrine Factors that Regulate FSH

Autonomous control of FSH production within the pituitary was initially revealed by the discovery of two related proteins: activins and inhibins (19, 20). They were first isolated from follicular fluid and identified based on their opposing actions on FSH secretion in pituitary cell cultures; inhibin blocks whereas activin stimulates FSH release (20, 21). The two proteins function differently where activin is produced within the pituitary gland to act as an autocrine factor, while inhibin is produced primarily by the gonads where it has an endocrine role in regulating FSH and other activin dependent genes [Figure 2; (4, 10)] . In addition to activins and inhibins, other members of the TGFβ superfamily including BMPs (BMP6, 7, and 15) were recently shown to be locally produced within

the pituitary and able to stimulate FSH production (10, 13, 15, 22). The activin/inhibin purification efforts also identified a glycoprotein, follistatin (FS), which was shown to antagonize activin by binding with high affinity (23). The discovery of follistatin helped characterize the autocrine/paracrine factors produced by pituitary cells. Studies have shown that cultured pituitary cells synthesize and secrete FSH in the absence exogenous factors, and addition of follistatin to cultured pituitary cells inhibits basal FSHβ expression and overall FSH production by 75% (24, 25). The data showed that pituitary cells are producing autocrine/paracrine factors including activins, BMPs, and possibly unidentified factors that maintain FSH production (10, 12, 13, 15).

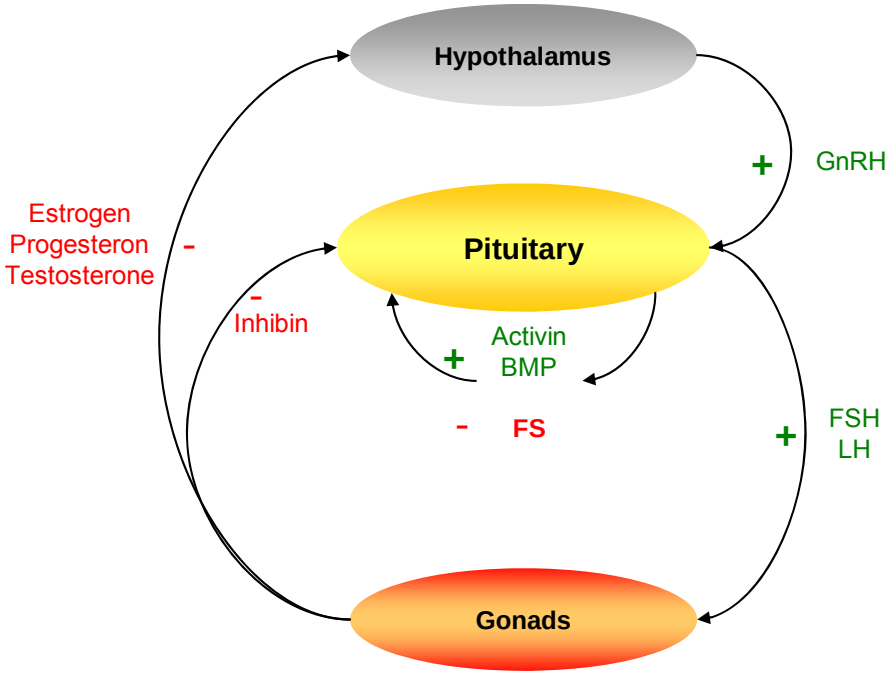


Figure 2: Schematic representation of hypothalamic-pituitary-gonadal axis showing positive and negative regulators of gonadotropin hormone gene expression. GnRH is synthesized in and released form the hypothalamus, where it travels to the pituitary and binds to GnRH receptor on gonadotropes. GnRH triggers the synthesis and ultimately the secretion of LH and FSH into the vascular system. Testosterone, estrogens, and progesterone can negatively regulate gonadotropin synthesis directly at the pituitary and via down regulation of hypothalamic GnRH secretion. Activins and BMPs are secreted within the anterior pituitary and stimulate FSH production. Inhibin is produced by the gonads and within the anterior pituitary to antagonize the effect of activin. Follistatin produced within the anterior pituitary bioneutralizes activins and BMPs therefore preventing induction of FSH production.

2. Cellular Models for Studying Gonadotropes:

The pituitary gland is composed of anterior and posterior pituitary lobes. To date at least five cell types form the anterior pituitary lobe which includes corticotropes, thyrotropes, somatotropes, lactotropes, and gonadotropes. Gonadotropes are identified by expression of FSH and LH, comprising about 3-10% of all pituitary cells (26). Studying the role of autocrine and individual paracrine factors has been difficult without separating gonadotropes from their surrounding cells. Furthermore, paracrine factors from other cell types provided an obstacle to studying the solitary role of factors regulating gonadotropes such as activins, BMPs, and others.

2A. LβT2 Immortalized Gonadotrope Cell Line

In an attempt to characterize gonadotropes, a transformed cell line of gonadotropes was developed. The cell line was derived from a transgenic mouse that carried a transgene composed of the LHβ promoter and the oncogenic SV40 large T-antigen. Gonadotropes in these mice expressed the oncogene during embryogenesis and some of the gonadotropes became permanently transformed [Figure 3]. The transformed cell line was named LβT2 (27). The LβT2 cell line became a useful model for studying gonadotropes since it mimicked primary cells in regulating FSH and LH (28-30). The LβT2 cells also showed an identical pattern of expression of the FSHβ subunit to primary gonadotropes, where activin and BMP treatments stimulate expression of the FSHβ subunit (13, 24). LβT2 cells have also been shown to express necessary signaling machinery to regulate FSHβ expression (28). Therefore, the cell line has provided a useful model for studying signaling pathways in gonadotropes and promoter elements required for FSHβ regulation.

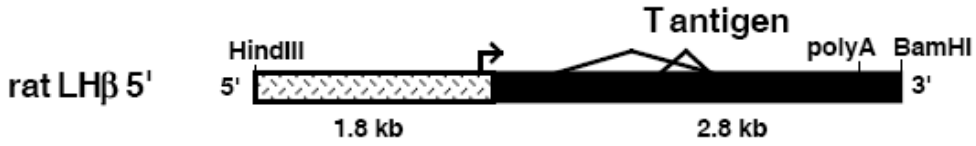


Figure 3: Structure of the LHβ T antigen transgene used to generate LβT2 cells [adapted from (27)].
The transgene contained 1.8 kb of the rat LHβ promoter to direct cell specific expression of the oncogene SV40 antigen to gonadotropes in the pituitary. The SV40 fragment contained the protein coding region for large and small T antigens, the translation initiation and transcription termination sites.

2B. Transgenic Approach for Studying Regulation of FSHβ

Several transgenic mouse models have been used to study the regulation of the FSHβ gene. One of the early transgenic mouse models was developed using a 10-kb region of the human FSHβ gene (4.3 kb of the 5'-promoter region, the complete coding region, and 2 kb of the 3'-flanking sequence). The construct was expressed exclusively in pituitary gonadotropes and regulated similarly to the endogenous mFSHβ gene, as detected by using an antibody specific for the human FSHβ subunit (31-33). However attempts to develop transgenic mice using 4.3 kb of the human FSHβ promoter to express a reporter gene specifically in gonadotropes were not successful.

Our laboratory, being interested in FSHβ regulatory elements, developed transgenic mice that harbor the construct made by cloning -4741 to +759 bp of the 5'-promoter region of ovine FSHβ gene into a luciferase reporter gene named oFSHβLuc [Figure 4A]. These transgenic mice showed pituitary specific luciferase expression (24). Another important finding with all these mice was that gonadectomy of males, which results in increase of FSH production due to relieve of inhibitory gonadal hormones, resulted in 2- to 8- fold

increase in luciferase activity. Ovariectomy of transgenic females, which also results in increased FSH production, caused 2- to 27-fold increase in luciferase activity compared to control mice. The transgenic female mice also showed normal cyclicity of luciferase expression (24). It became evident that the in vivo expression of luciferase mimicked the expression of endogenous FSHβ subunit.

Next, the role of pituitary factors was tested in vitro on cultured pituitary cells from the transgenic mice. Follistatin (250 ng/ml) and inhibin (10 ng/ml) treatment of cultured cells decreased luciferase expression by 61-82% and 59-79%, respectively, similar to their suppressive actions observed in pituitary cell cultures from non-transgenic mice or rats. Furthermore, activin induced expression of oFSHβLuc by 8-fold in the transgenic mice (24, 25). So, for the first time a transgenic animal model became available that couples all the regulatory elements required for both, tissue specific expression and regulation of FSHβ reported by expression of the luciferase gene.

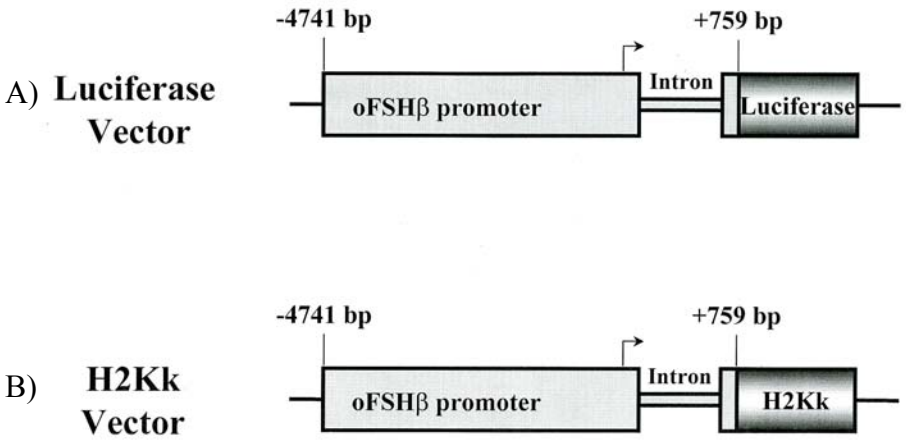


Figure 4: oFSHβ constructs direct cell specific expression
A) Transgenic mice harboring the ovine FSHβ (oFSHβ) promoter plus first intron (from -4741 to +759) linked to a luciferase reporter gene (oFSHβLuc) were generated to direct tissue-specific expression in vivo and serve as a model for studying hormonal regulation of the FSHβ gene. B) Using the same construct transgenic mice were developed that had H2Kk cell surface antigen linked to oFSHβ promoter targeting gonadotrope specific expression to allow purification of primary gonadotropes.

2C. Purified Primary Gonadotropes

A different approach was employed in our laboratory to study primary gonadotropes. The method developed to isolate gonadotropes relied on the ability of the cloned ovine FSHβ promoter (-4741 to +759) to target cell specific expression selectively to gonadotropes. The oFSHβ cloned promoter was linked to the coding region of a cell surface antigen named H-2Kk forming oFSHβH2Kk construct that was used to generate transgenic mice harboring the construct [Figure 4B]. The gonadotropes were purified from dispersed transgenic mouse pituitary cells using anti-H2Kk microbeads and cells were sorted over a column in a magnetic field [Figure 5; (34)]. The purified gonadotropes have been used to study autocrine factors, and also have been a useful tool to study the role of activin, BMPs, and follistatin on pure gonadotrope cultures. In the

future, studies will be performed to asses any differences between purified gonadotropes and LβT2 gonadotrope cell line.

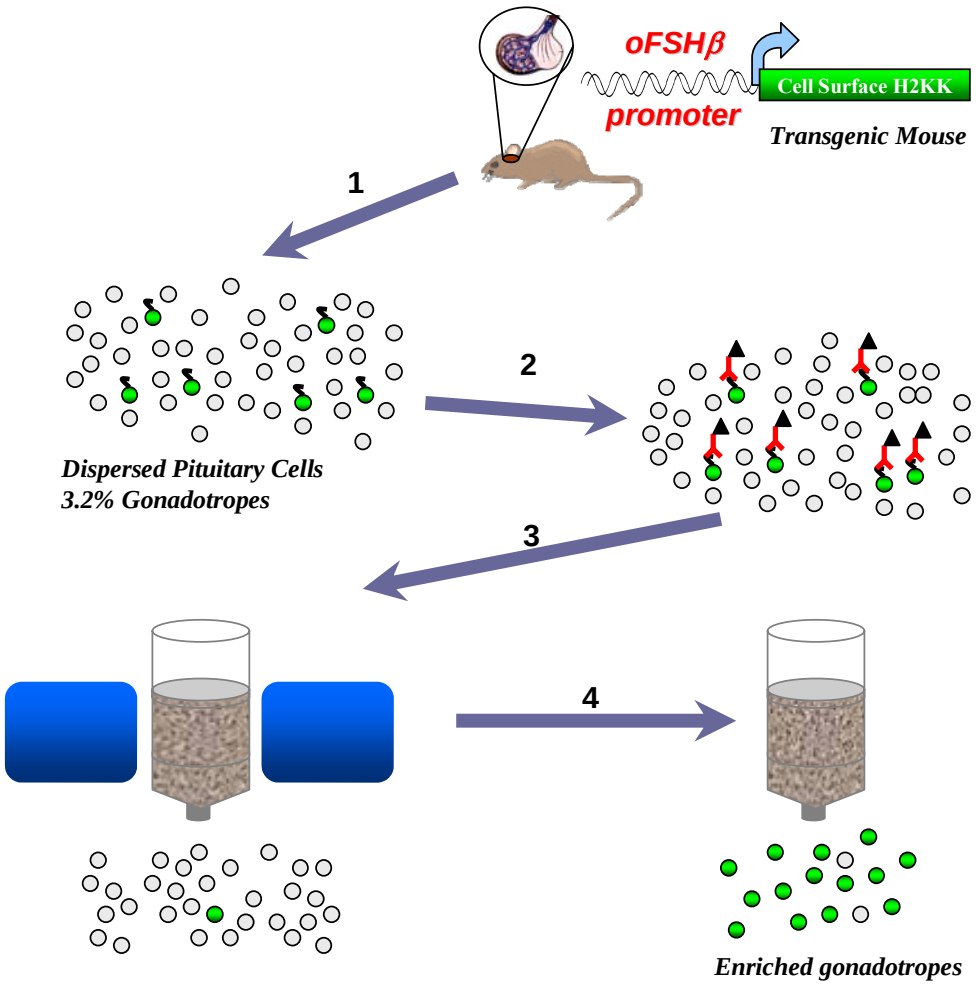


Figure 5: Positive selection of gonadotropes
Dispersed pituitary cells [1] from transgenic mice harboring the oFSHβH2Kk construct were labeled with anti-H2Kk para-magnetic microbeads [2]. The cells were then passed over a column in a magnetic field [3], and gonadotropes were retained on the column while other cell types were eluted in the flow through fraction. The column was then removed from the magnetic field and gonadotropes were eluted as the positive fraction [4].

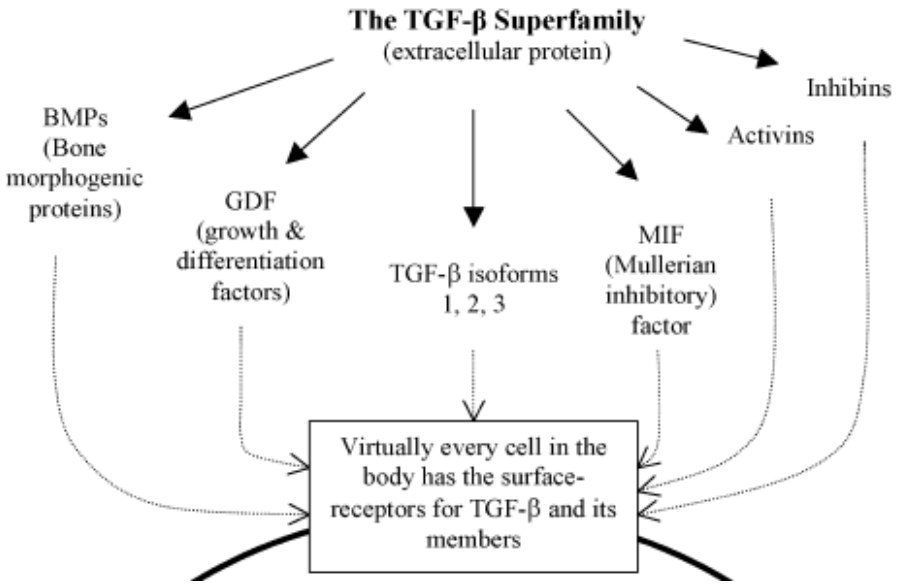


Figure 6: Overview of major TGFβ superfamily members [adapted from (35)]
The TGFβ superfamily is comprised of subfamilies that include BMPs, GDFs, TGFβs, MIF, Activins, Inhibins, and others.

3. Activin is the most potent stimulator of FSHβ expression:

The importance of activins and inhibins in the control of reproductive function is substantiated by numerous studies and clinical observations (36). Activins along with inhibins were initially purified from gonadal fluids and characterized based upon their ability to modulate FSH secretion from pituitary gonadotropes (20, 21, 34). Since their discovery, it has been shown that activins are produced in different tissues including the pituitary gland where they are involved in regulating different physiological functions (36). Several studies have confirmed that bioactive activins are not detected in circulation and instead act as local autocrine/paracrine factors within a specific tissue (10).

Activins belong to the transforming growth factor β (TGFβ) superfamily of secreted proteins sharing a conserved seven cystiene C-terminal motif common to all family members [Figure 6; (37)]. Activin, similar to members of the TGFβ super family, is produced as a precursor protein that is cleaved and secreted as a bioactive dimer. Three active isoforms of activin have been identified in mammalian systems and named based on subunit assembly: activin A (β_A-β_A), activin B (β_B-β_B), and activin AB (β_A-β_B) [Figure 7; (38)] . No differences have been shown between the activin isoforms, and studies have primarily used activin A because it was the first commercially available isoform (39).

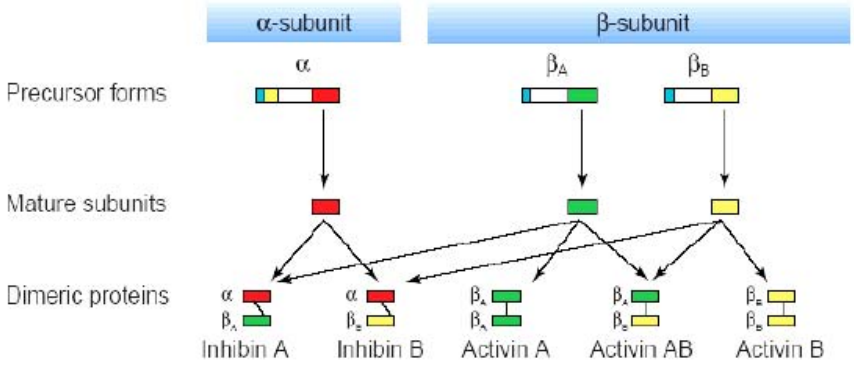


Figure 7: Activin subunit assembly [adapted from (38)].
Both α- and β-subunits of inhibin and activin are synthesized in precursor forms, and are then cleaved to mature subunits. Heterodimerization of the α- and β-subunits forms "inhibin" protein, whereas homodimerization of the β-subunits results in activin. The type of protein formed depends on the type of β-subunit.

3A. Activin Induction of FSHβ

Activin has been shown to have a wide range of biological activities, and most notably its role as a specific stimulator of FSHβ expression, without affecting LHβ or αGSU (10). Carrol et al. have shown that activin treatment of cultured pituitary cells for 72 hours stimulated FSH secretion by 2.5-fold, while FSHβ mRNA was simultaneously stimulated 4-fold (11). In vivo studies using ovariectomized female rats have shown that subcutaneous injection of activin (500 μg/kg) increased serum FSH by 1.5-fold and increased pituitary FSHβ mRNA by 2.7-fold (40). The impact of activin treatment on induction of FSHβ was most clearly shown in a study using rat pituitary cell perifusion system. The perifusion system allowed constant removal of antagonizing paracrine factors produced by pituitary cells. Administration of activin to the perifusion system stimulated FSHβ mRNA by up to 25-fold (33).

More recently, the availability of LβT2 transformed gonadotrope cell line and cloning of the FSHβ promoter from the sheep, human, and rat have permitted a more detailed analysis of the mechanisms underlying the actions of activins in gonadotropes. Activin treatment (24h) of cultured LβT2 cells stimulated endogenous mouse FSHβ mRNA expression by 60-fold (41). Transfection studies utilizing the cloned oFSHβLuc constructs have shown that activin treatment (24h) stimulated oFSHβLuc expression by 6-fold (28). From in vivo and in vitro studies, it is now clear that the stimulatory actions of activin on FSH synthesis and secretion are mediated through stimulation of the FSHβ gene specifically.

3B. Role of Activin B in the Pituitary

Activins clearly stimulated FSH synthesis in the pituitary, but whether activins are synthesized within the pituitary gland was not clear until Roberts et al. showed the localization of activin B homodimers ($\beta_B\beta_B$) within pituitary gonadotropes (42). Corrigan et al. showed that rat pituitary cultures treated with bioneutralizing mouse monoclonal anti-activin B for 72h showed a 50% decrease in basal FSH secretion. The antibody also decreased FSHβ mRNA expression by about 63% compared to control levels (12). DePaolo et al. have used ovariectomized hypophysectomized pituitary grafted (OVX H/G) rats as an in vivo model to test the intra pituitary role of activin B in the absence of neuroendocrine control (GnRH). Injection of 4.2 mg of anti-activin B MAb for 12h decreased FSH secretion by 57% compared to control levels (43). The data confirmed the hypothesis that activin B is secreted within the pituitary and acts as an autocrine stimulator of FSH synthesis.

4. Activin Signaling Pathway:

Activin belongs to the TGFβ superfamily that comprises a large number of structurally related polypeptides each capable of regulating a wide range cell specific processes including proliferation, differentiation, motility, cell death, and many others (10). TGFβ superfamily members share a general mechanism by which they activate receptors at the cell surface and transduce signals to target genes [Figure 8]. The signal is initiated by ligand binding to a serine/threonine kinase receptor complex. The activated receptor complex transduces the signal downstream either by activating Smad proteins signaling and/or by initiating a mitogen activated protein kinase (MAPK) signaling cascade. Both signaling pathways can independently regulate different genes within the same cell, while in other systems cross-talk is observed between the two pathways to regulate the same gene (27, 37, 44).

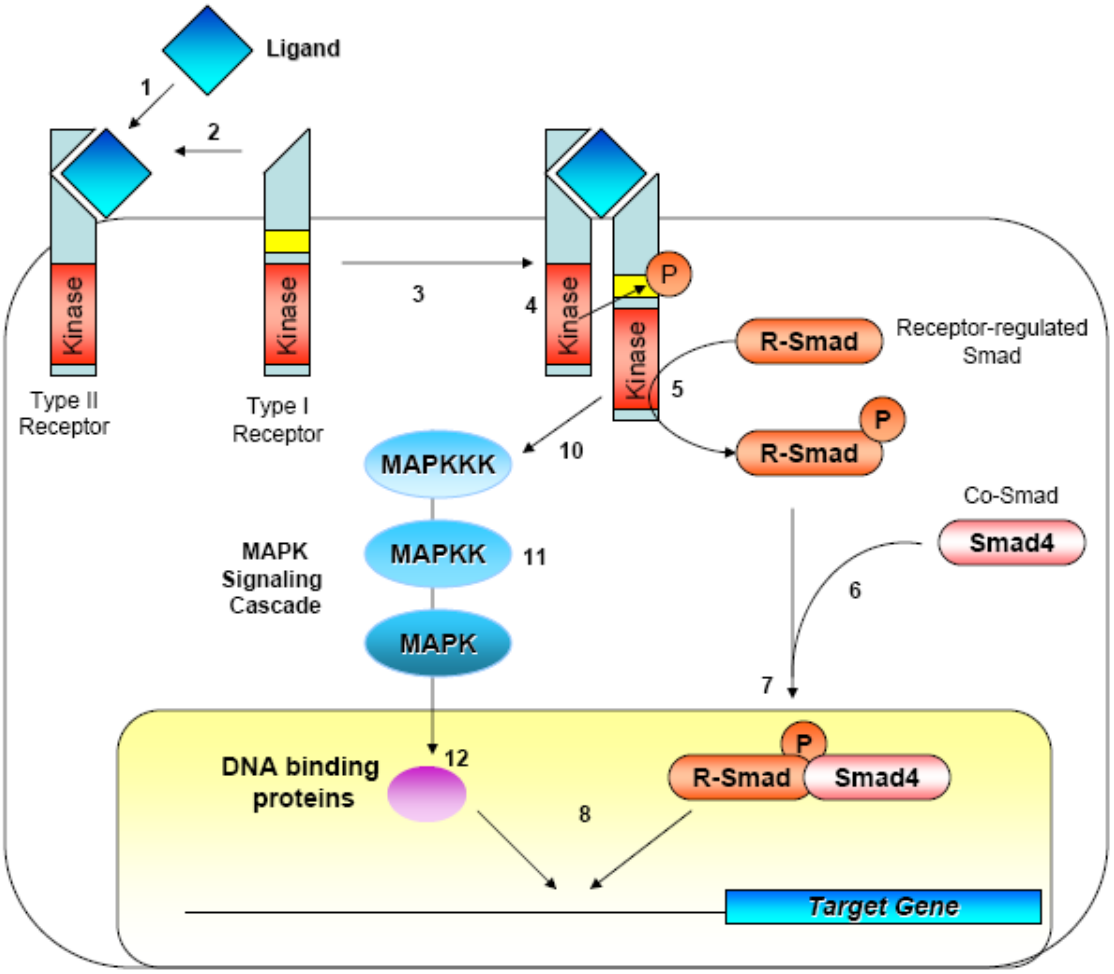


Figure 8: The activin/TGFβ Smad pathway.
Steps of the signaling pathway are numbered as follows: Binding of activin or TGFβ to its type-II receptor [1] which recruits type-I receptor [2] leading to the formation of the receptor complex [3] allowing transphosphorylation between the receptors [4]. The type-I activated receptor can phosphorylate receptor-activated Smad (R-Smad) [5]. The activated R-Smad then associates with a partner protein named Smad4 (Co-Smad) and moves in to the nucleus [6 and 7]. Once in the nucleus the Smad complex can bind to promoter elements and associate with transcription factors, co-activators, co-repressors [8]. Members of the MAPKKK family can associate with the type-I receptors and are activated [10]. Activation of the MAPKKK initiates the MAPK signaling cascade where the MAPKKK phosphorylates downstream MAPKK proteins, which in turn phosphorylate MAPK proteins [11]. The activated MAPK proteins then phosphorylate DNA binding proteins that can associate with the promoter to induce or suppress expression of the target gene [12].

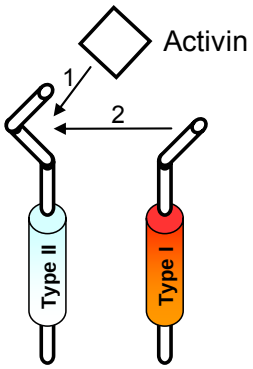
5. Activin Receptors:

Activin and related TGFβ superfamily members signal through a family of serine/threonine kinase transmembrane receptors. The receptors are classified into two subfamilies: type I receptors and type II receptors based on structural and functional properties. The type I and type II receptors are glycoproteins of 55kDa and 70kDa, respectively. Activin initiates the signaling pathway by binding in a sequential fashion to the type II receptor. The activin bound type II receptor is then able to recruit the type I receptor forming the activated receptor complex (37).

5A. Type I and Type II Activin Receptors

Activin initiates signaling by binding to the activin type II receptor. There are two isoforms of the activin type II receptor: ActR-II and ActR-IIB. The type II receptor has a kinase domain and is able to autophosphorylate itself at basal conditions. Thus, activin binding to the type-II (ActRII or ActRIIB) receptor initiates signaling by recruiting the type-I receptor. The activin type I receptor is not phosphorylated at the basal state. However, upon activin binding to the activin type II receptor, the activin type I receptor is recruited to form a receptor complex formed by the activin-bound type II receptor and the type I receptor. Activin holds both receptors in close proximity, and the type II receptor trans-phosphorylates the type-I receptor leading to its activation. The type-I receptor isoforms are: activin receptor-I (ActR-I) also known as activin-like kinase 2 (ALK2), and a second isoform which is primarily found to mediate activin signaling is activin receptor-IB (ActR-IB) also known as ALK4. The stoichiometry of the activated

receptor complex involves the association of two activins with two activin type II receptors (ActRII or ActRIIB) and two activin type I receptors (37, 38, 45).



Ligand	Receptor II	Receptor I
Activin	ActR-II ActR-IIB	ActR-IB ActR-I

Figure 9: Activin receptor type I and type I Isoforms.
Signaling starts by sequential binding of activin to the type II receptor leading to recruitment of the type I receptor forming a receptor-ligand complex. The receptor complex can be formed by different combination of Type II and Type I receptor, but the mechanism is not yet clearly defined.

5B. Activin Type-I Receptor Initiates Downstream Signaling

Activin specific stimulation has been shown to be mediated through the activin type I receptor (22, 37). Thus the activin type I receptor has been shown to activate the Smad signaling pathway or initiate the MAPK signaling cascade, while the activin type II receptor does not play a role in signaling downstream of the receptor (37, 44). In an attempt to further understand the role of ActR-IB as the main component of the receptor complex, a constitutively active kinase mutant was constructed by a single mutation of threonine-206 to aspartic acid in the activin type IB receptor (ActR-IB T206D). The mutation allows ActRIB (T206D) to be constitutively independent of activin or the type-

II receptor (22). The data confirm that ActR-IB acts as downstream mediator of activin signaling. Studies with the gonadotrope cell line LβT2 show that ActR-IB is the receptor mediating activin induction of FSHβ expression, and the study also showed that transfecting the constitutively active receptor ActR-IB(T206D) stimulated FSHβ mRNA by 86-fold (41). Therefore, the constitutively active receptor ActR-IB(T206D) has been used to initiate the activins signaling pathway in the absence of activin, and many studies have focused on identifying intracellular mediators that are activated in cells treated with activin or transfected with the constitutively active receptor ActR-IB(T206D) (41).

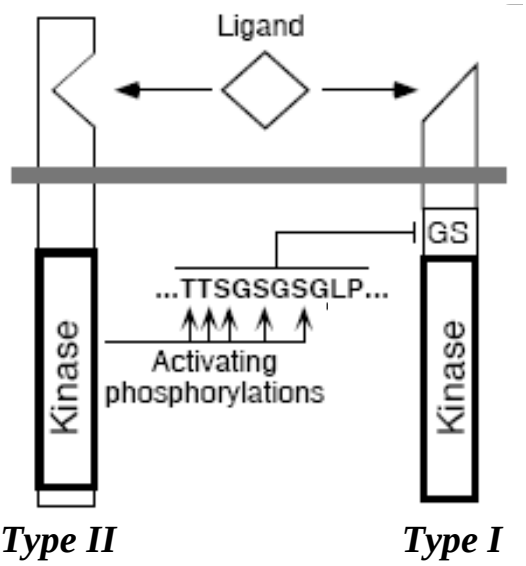


Figure 10: Transphosphorylation of the activin receptor complex [adapted from (37)] The characteristic GS sequence motif of the ActR-I is shown, indicating sites that are phosphorylated by the ActR-II leading to activation of ActR-I.

6. Smad Proteins: Mediators of Activin Signaling

Smad proteins, discovered through genetic studies in *Drosophila* and *Caenorhabditits elegans (C. elegans)*, have proven to be pivotal intracellular mediators of TGFβ superfamily members in many biological systems. The name originates from a fusion between Drosophila *mother against dpp (Mad) and C. elegans Sma*. The Smad family consists of proteins with molecular weights ranging from 42 kDa to 65 kDa. The role of Smad proteins divides them into three distinct subfamilies: receptor regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads) [Figure 11; (37, 45, 46)].

6A. Role of Smads in Mediating TGFβ Superfamily Signaling Pathway

Upon ligand binding and activation of the receptor complex, activated type I receptor kinases specifically and transiently interact with and phosphorylate particular R-Smads. Subsequently the activated R-Smads recruit Co-Smads and translocate to the nucleus. In the nucleus Smad complexes can bind DNA directly or indirectly through interactions with other DNA-binding proteins (37).

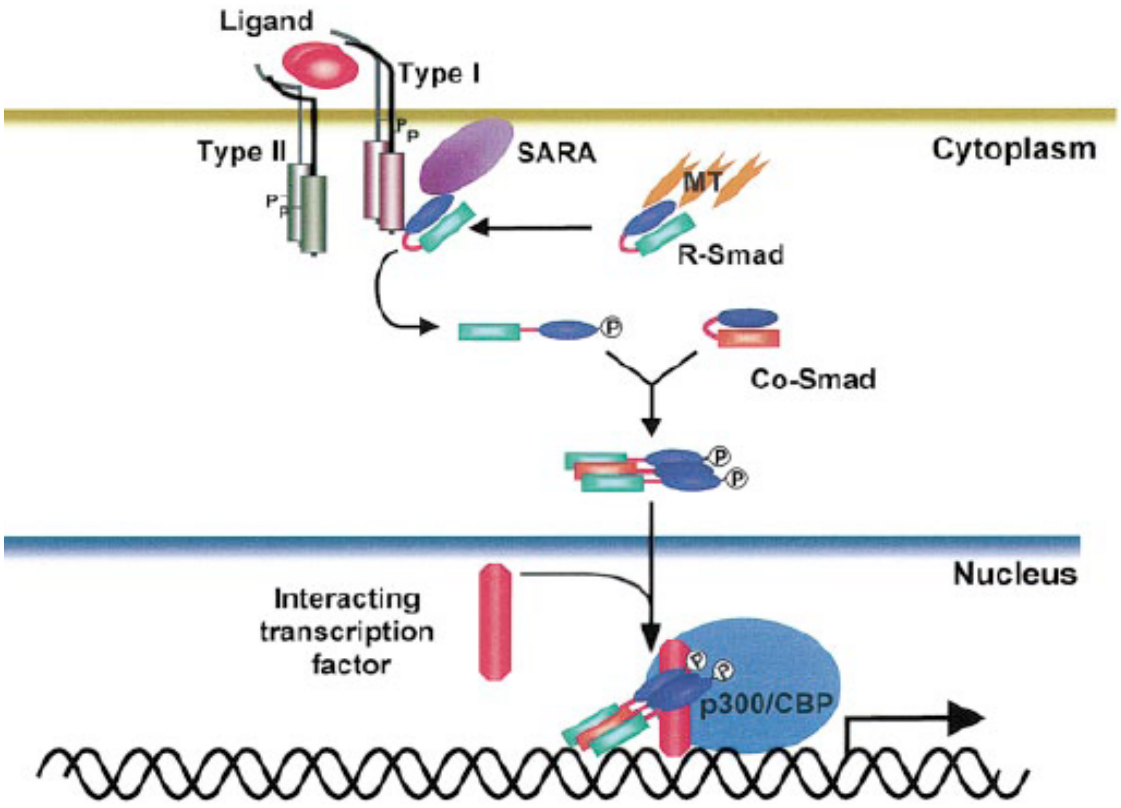


Figure 11: Activation of R- and Co-Smads [adapted from (45)].
Upon ligand binding, the activated receptor complex transduces an intracellular signal initiated by the activated type I receptor phosphorylating R-Smads. The activated R-Smads form a heteromeric complex with Co-Smads that translocates into the nucleus. In the nucleus the R-Smad/Co-Smad complex bind promoter elements and associate with transcriptional co-activators and interacting transcription factors to initiate cell specific expression.

6B. Smad Families and Subfamilies

The R-Smads can be further divided into two groups: BMP-Smads and TGFβ/activin-Smads [Figure 12; (47)]. BMPs specifically activate Smad1, Smad5, and Smad8 through type-I BMP receptors. Activin and TGFβ activate Smad2 and Smad3 through the activated TGFβ and activin type-I receptors. However, it is still unclear how the receptor preferentially selects for Smad2 or Smad3 to activate a specific gene (48). Co-Smads are

the second subfamily with only Smad4 being identified. The name dictates the function since Smad4 is expressed and interacts with activated R-Smads forming a R-Smad/Smad4 complex [Figure 11]. The complex can then translocate to the nucleus to bind DNA or transcription factors. Smad6 and Smad7 belong to the subfamily of inhibitory Smads (I-Smads), and they function as a negative feed back regulation preventing R-Smad activation and association with Smad4 (37, 45, 47, 49).

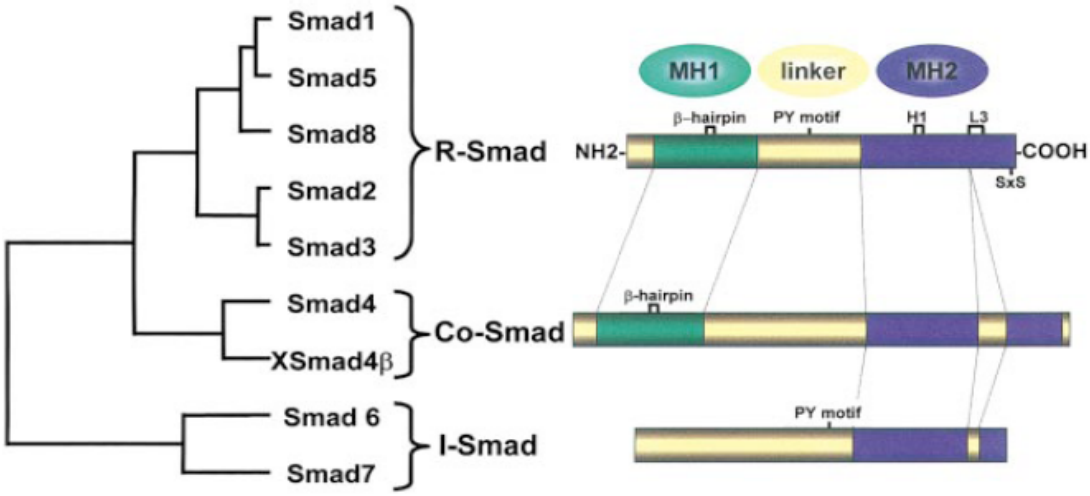


Figure 12: The Smad Family [adapted from (45)].
Phylogenetic tree of the Smads and their structures. R-Smads include Smad1, Smad2, Smad3, Smad5, and Smad8. All R-Smads share an MH1 domain, linker region, and an MH2 domain containing SSXS phosphorylation sites. R-Smads 1, 5, and 8 are activated by BMPs, while Smads 2 and 3 are activated by activins and TGFβ 1-3. Smad4 belongs to the Co-Smad subfamily. The inhibitory or I-Smad subfamily includes Smad6 and Smad7 which lack the MH1 domain.

6C. Structural Motifs of Smads

R-Smads and Co-Smads are highly homologous at their N- and C-termini, which are termed MAD homology-1 (MH1) and MH2 domains, respectively [Figure 12]. Both domains are separated by a divergent proline-rich linker region of variable length. R-Smads interact with the type-I receptor via the L3 loop within its MH2 domain (45). Upon phosphorylation of R-Smads, they dissociate from the receptor and are able to interact with Co-Smad4 through interaction via the MH2 domain. Once in the nucleus, only Smad3 and Smad4 are able to bind specific DNA sequences through their MH1 domain (46). All R-Smads can interact with transcriptional coactivators via their MH2 domain allowing them to be part of the transcriptional machinery [Figure 12; (37, 45, 46)]. I-Smads contain a conserved MH2 domain that is also able to interact with type-I receptor to inhibit activation of R-Smads [Figure 12; (45)].

7. Signaling Through Smads:

Upon ligand binding the activated receptor complex transduces the signal by activating the Smad signaling pathway. In the basal state, Smads exist in an auto-inhibitory conformation formed by interaction between the MH1 and MH2 domains. Upon ligand activation of the receptor complex, the type-I receptor kinase phosphorylates specific R-Smads. The phosphorylated R-Smads associate with Smad4 and translocate to the nucleus, where they bind to promoter elements or associate with transcription factors [Figure 13; (47)].

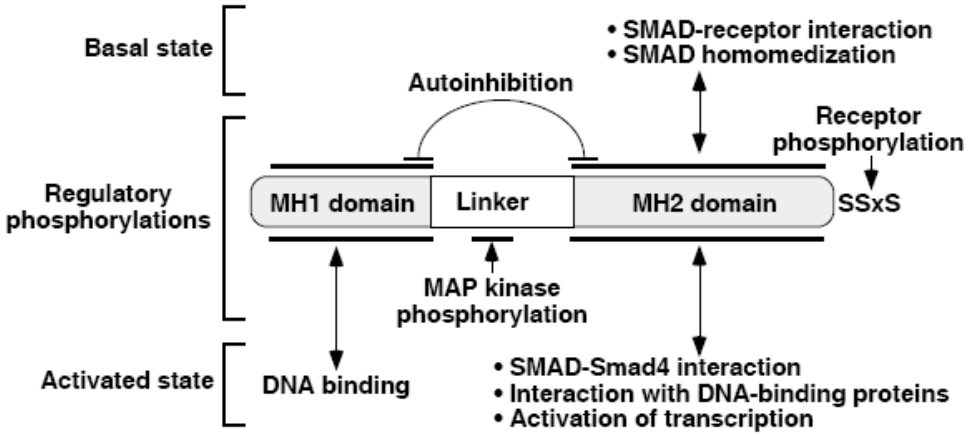


Figure 13: R-Smads Structure [adapted from (37)].
In the basal state, R-Smads adopt an autoinhibitory conformation through the interaction of the MH1 and the MH2 domains. R-Smads interact with the type-I receptors via the MH2 domain and become activated by receptor-mediated phosphorylation at the C-terminal SS(X)S motif. Activated R-Smads associate with Smad4 and transcriptional co-activators via the MH2 domain. The MH1 domain of Smad3 is also able to bind DNA. R-Smads can also be regulated by the MAPK pathway via phosphorylation of the linker region

7A. Smads as a Receptor Substrate

Upon ligand activation of the receptor complex, R-Smads are phosphorylated at their C-terminal motif serine residues (SSXS) [Figure 13]. Upon phosphorylation of R-Smads, the affinity decreases between the type I receptor and R-Smads. This results in a dissociation of the complex between type I receptor and R-Smads. The free phosphorylated R-Smad has an affinity for Smad4 and associates with it to form a R-Smad/Smad4 heteromeric complex which translocates to the nucleus. In recent reports however, it has been shown that certain cell types show constitutive nuclear translocation of Smad2 and Smad3 in a ligand-independent fashion (45, 47).

7B. Dominant Negative Smad2 and Smad3

The importance of the three conserved serine residues was confirmed using dominant negative Smad2 (3SA) and dominant negative Smad3 (3SA), in which the three serine residues are mutated to alanine at the C-terminal end. The mutation prevents activation of Smad2 and Smad3, and over expression of the dominant negative Smad2 (3SA) or Smad3 (3SA) blocks activation of endogenous Smad2 and Smad3, respectively (50).

Another dominant negative form of Smad3 was constructed by substituting aspartate at position 407 to glutamate generating Smad3 (D407E). The mutation allows Smad3 (D407E) to associate with the type-I receptor, but prevents dissociation of Smad3 (D407E) from the receptor blocking the receptor's active site. Transfection of Smad3 (D407E) has been shown to block activation of endogenous Smad2 and Smad3 (51). Although recent studies have concluded that Smad3 could play a role in activin mediated stimulation of FSHβ gene, results presented later in this study from our laboratory using the dominant negative Smad3 (3SA) and Smad3 (D407E) indicates that Smad3 is not a mediator of FSHβ stimulation by activins (52).

7C. Smad Binding Elements

Once in the nucleus, Smad3 and Smad4 have the ability to bind DNA through interaction of their MH1 domains and the specific DNA sequence (5'-AGAC-3') termed the Smad-binding element (SBE). Although Smad3 and Smad4 can bind SBE on the promoter, the affinity for DNA binding is low and the complex needs to be stabilized by association with other co-activators, co-repressors, or transcription factors [Figure 15]. Smad2 however is unable to bind DNA directly and can interact with Smad4 to associate with

promoter elements or can bind transcription factors bound to DNA through protein-protein interactions (37, 45, 47).

7**D. Smad Associated Transcriptional Complexes**

Smad2 and Smad3 are expressed in almost all cell types and thus the cell specific response is the result of interaction with cell specific transcription factors, coactivators and corepressors that denotes regulation of specific genes (44). Smad2 and Smad3 have been shown to interact with transcription complexes through their MH2 domain. *Xenopus* forkhead activin signal transducer (FAST-1) was the first transcription factor partner for Smads to be identified (37). Subsequently, a large number of transcriptional factors, co-activators, and co-repressors have been found to associate with Smads [Figure 15] and regulate transcriptional activity of activin responsive genes (44, 53).

Recent data show that Smad2 and Smad3 are not only involved in activation a specific gene, but can also cause repression of a specific gene. In the activated genes, Smad2 and Smad3 interact with coactivators such as p300 and CBP, which promote transcription by facilitating the interaction of Smads with basal transcription machinery or through their intrinsic (or recruited) histone acetylase activity which loosens chromatin structure [Figure 14, (46, 54)].

Repression of a gene by Smads can occur by their ability to interact with transcriptional corepressors. The structurally related proto-oncogene products Ski and SnoN were shown to act as transcriptional corepressors for Smads. Ski can also recruit HDACs

indirectly to repress expression. In addition, Ski can also inhibit transcription by competing with CBP/p300 for Smad interaction (54).

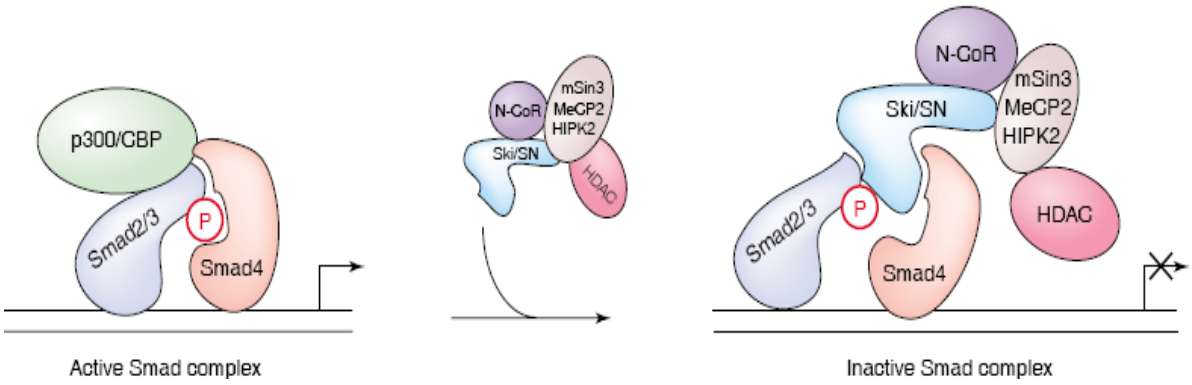


Figure 14: Mechanism or repression of the Smad proteins by Ski and SnoN [adapted from (54)] Smad2 and Smad3 can act as bimodal modulators of gene expression. In the activated state Smad2 and Smad3 interact with coactivators that recruit histone acetylase leading to expression of the gene. In the repressed state, Smad2 or Smad3 interact with transcriptional co-repressors that recruit histone deacetylase leading to repression of the gene.

8. The Roles of Smads in Gonadotropes:

Recent reports suggest that Smad2 and Smad3 are intracellular mediators of activin in gonadotropes. Studies in LβT2 cells have shown that over expression of Smad3, but not Smad2 induced basal expression of the FSHβ gene by 9-fold (55). Treatment of the Smad3-transfected LβT2 cells with activin increased by 40-fold above the basal levels. In addition, treatment of LβT2 cells with activin increased expression of FSHβ by 4-5 fold (55). Thus transfection of Smad3 only increased basal expression by 9- fold but does not enhance activin induction of FSHβ gene (stimulation from 9-fold to 40-fold is still about 4-fold). Although initial data on Smad3 studies suggested a role of Smad3 in mediating activin induction of FSHβ, current data studies presented here suggest that a

Smad-independent pathway is the primary mediator of activins signaling in gonadotropes (52, 55).

9. The FSHβ Promoter Contains Smad Binding Elements:

Deletion studies in the FSHβ promoter have identified a region that is required for activin induction of the FSHβ gene, and is commonly referred to as the activin response element (ARE) (55-57). The same studies have also identified SBE in both the rat-FSHβ and ovine-FSHβ promoters (55, 56). However, Smad3 and Smad4 were observed to be bound to the SBE on both rat-FSHβ and ovine-FSHβ at basal conditions in the absence of activin (55, 56). The data show that Smad3 and Smad4 remain bound to promoter elements as part of the transcriptional complex that drives basal expression, and it is the transcriptional co-activators activated by a Smad-independent pathway are the primary inducers for FSHβ expression.

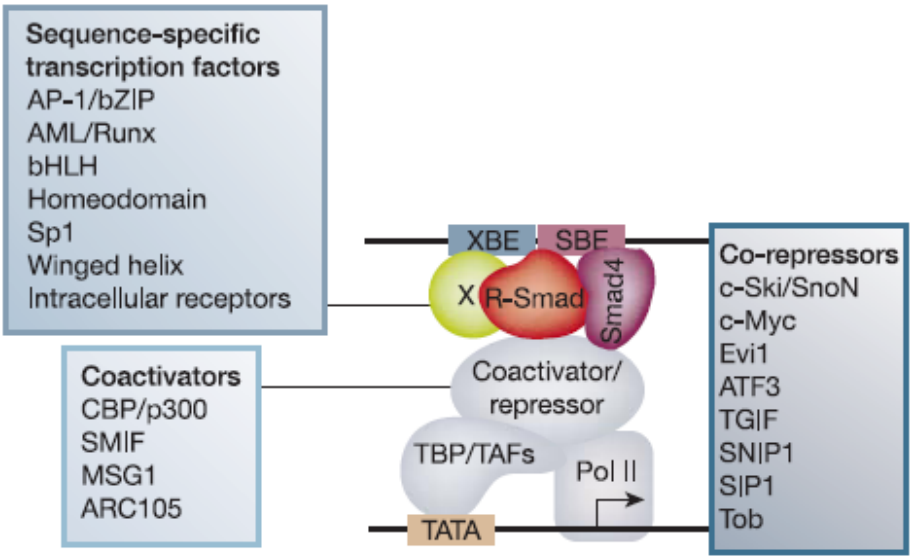


Figure 15: R-Smad/Smad4 complex cooperates with co-activators/corepressors [adapted from (44)].
R-Smads/Smad4 can interact directly with the essential co-activators such as CBP or p300. Corepressors can also interact with R-Smad/Smad4 to down regulate Smad-mediated transactivation such as c-Ski and SnoN. The activated transcription factors or coactivators that interact with R-Smad/Smad4 are believed to trigger the specific gene expression by having higher affinity displacing corepressors.

10. Role of MAPKinases in TGFβ Family Signaling:

Although Smad-dependent pathways were known to be the core of TGFβ family signal transduction, there is growing evidence that TGFβ family members can elicit a response through a Smad-independent signaling pathway (44). TGFβ activated kinase 1 (TAK1) is a member of the MAPKKK family and has recently been shown to be a critical mediator in the signaling pathway stimulated by activin, TGFβ, and BMPs (1, 58-60).

10A. MAPK Signaling Cascade

Signaling of the MAPK family is organized hierarchically in three different steps. The signaling cascade initiates by activation of MAPKKK such as MLK, TAK1, and ASK1 kinases. Activated MAPKKKs can phosphorylate downstream MAPKKs such as MKK3 and MKK6, which then can activate downstream MAPKs such as p38-MAPK, ERK, and JNK (44, 61, 62). Transcription factors and gene specific co-factors are then phosphorylated to associate with the transcription complex driving gene expression (44).

10B. TAK1 Structure

The TAK1 kinase is composed of 579 amino acids with a putative N-terminal protein kinase catalytic domain and a 300-residue C-terminal domain [Figure 16]. The first 22 amino acids on the N-terminal domain have been shown to regulate activation of TAK1 (59, 63, 64). ATP binds at Lysine-63 within the kinase domain active site, and mutation of Lysine-63 to tryptophane results in a catalytically inactive TAK1(K63W) that has been used as a dominant negative inhibitor of endogenous TAK1 activation (65).

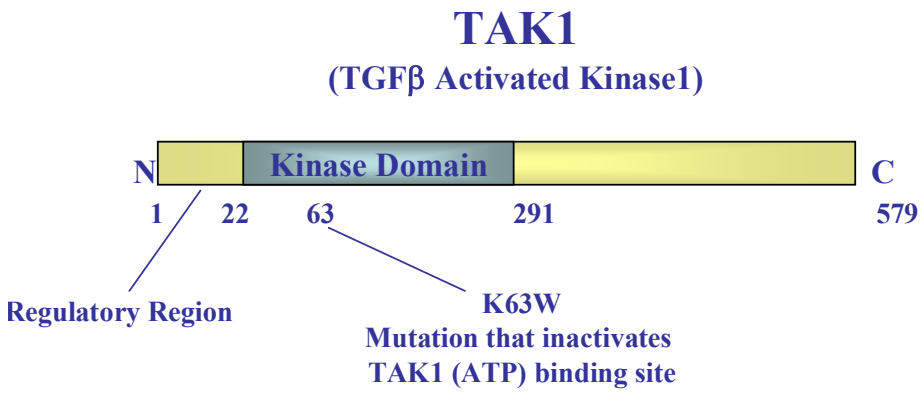


Figure 16: Schematic structure of TAK1 protein.
TAK1 is 579 amino acid protein with kinase domain located between amino acid 22 and 291. Lysine-63 is required for ATP binding and TAK1 kinase activity, and mutating lysine-63 to tryptophane prevents the activation of TAK1.

10C. TAB1, a TAK1 Binding Protein

In efforts to understand the mechanism of activation of TAK1 by TGFβ family members, a protein that partners with TAK1 was identified as TAK1-binding (TAB1) protein. TAB1 is a 504 amino acid protein having a molecular weight of 55 KDa that shows no marked similarities to known proteins. It was first identified using a two-hybrid system to identify proteins that interact with TAK1, and was also shown to associate with TAK1 in mammalian cells (64). TAB1 binds TAK1 at the C-terminal motif inducing a conformational change required for activation of TAK1 [Figure 17; (66)]

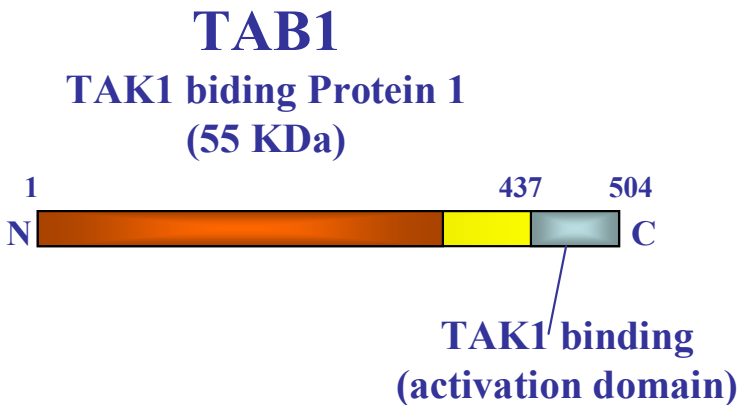


Figure 17: Structure of TAB1
TAB1 is 504 amino acid protein. It associates with TAK1 through a C-terminal motif composed of 68 amino acids. TAB1 is known to be associated with TAK1 in vivo, but it is not clearly understood how TAB1 binding is able to activate TAK1 kinase activity in response to ligand stimulation.

11. TAK1 Activation:

Activin, TGFβ, and BMPs have been shown to activate TAK1. Although autophosphorylation mediated by intramolecular reaction has been implicated in the activation of TAK1, the actual mechanism of how the receptor activates TAK1 is still unclear. Recent studies have highlighted that association of TAB1 is required for TAK1 activation. TAB1 is believed to induce a conformational change that allows TAK1 to autophosphorylate its activation domain (66, 67).

11A. TAK1 Activation by TAB1

Although endogenous TAK1 naturally associates with TAB1, TAK1 remains unphosphorylated in the absence of ligand (activin, TGFβ, or BMP) stimulation through the activated receptor complex (64, 68, 69). Therefore, binding of TAB1 to TAK1 is not sufficient to induce phosphoyrlation and activation of endogenous TAK1 in vivo. Co-transfection of TAK1 and TAB1 however activates TAK1 in the absence of ligand stimulation, suggesting that overexpression of TAK1 and TAB1 by-passes a putative inhibitory factor that blocks the activity of the endogenous TAK1-TAB1 complex (67, 70). These results suggest that ligand treatment may disrupt the inhibitory regulation thereby allowing TAK1 activation in the presence of TAB1.

11B. Studies using inhibitors of TAK1 kinase activity

To understand the role of endogenous TAK1 as mediator of activin and TGFβ signaling a dominant TAK1(K63W) was constructed. The catalytically inactive TAK1(K63W) has a lysine-63 residue, which is required for ATP binding, replaced by a tryptophane. Over

expression of TAK1(K63W) has been shown to act as a dominant negative inhibitor to prevent activation of endogenous TAK1 (65). A recent study also identified 5Z-7-Oxozeanol, a resocyclic lactone of fungal origin, as a specific chemical inhibitor of TAK1 activation. 5Z-7-Oxozeanol had no effect on the kinase activity of other members of the MAPKKK, and was shown to selectively inhibit TAK1 activation by competing for the ATP binding site required for TAK1 activation (71).

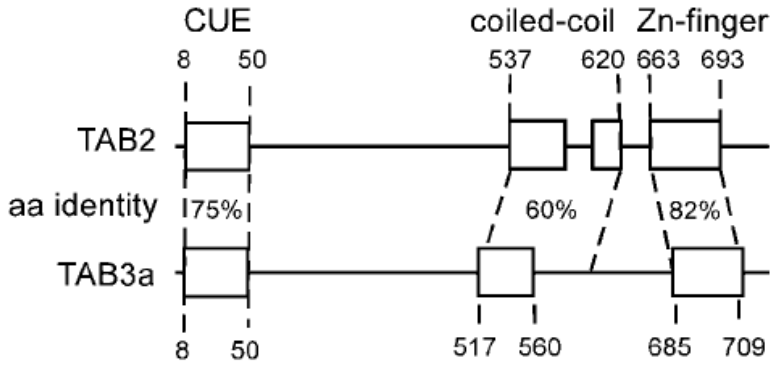


Figure 18: Sequence homology between TAB2 and TAB3 [adapted from (72)] Coiled-coil structure in TAB2 and TAB3 at the c-terminal share 60% sequence homology are required for interaction with TAK1.

11C. TAB2 and TAB3 Partner with TAK1

TAB2 was discovered shortly after identification of TAB1, TAB2 was found to associate with the c-terminal domain of TAK1 (69). Recently TAB3 was also identified and it was shown to associate with the c-terminal domain of TAK1 (68). TAB2 and TAB3 share structural similarities as shown in Figure 18. TAB2 and TAB3 are binding partners of

TAK1 and are believed to function as adaptor molecules linking TAK1 to upstream receptors (68, 73). It has been previously shown that TAK1 partners with TAB1 and either TAB2 or TAB3 forming TAK1/TAB1/TAB2 or TAK1/TAB1/TAB3 complexes [Figure 19; (68, 69, 72, 73)]. Over expression of TAB2 and TAB3 have been shown to induce TAK1 dependent genes, however the mechanism by which TAB2 and TAB3 regulate TAK1 is not clearly defined (67-70, 73).

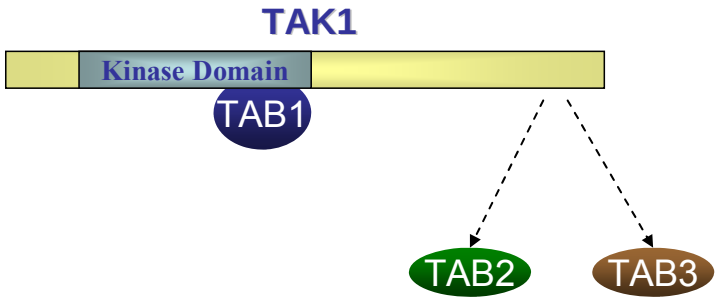


Figure 19: Schematic representation of TAB1, TAB2, and TAB3 binding to TAK1. TAK1 requires TAB1 to induce activation of TAK1 kinase activity. The binding of TAB2 or TAB3 to TAK1 occurs at the c-terminal end to form TAK1/TAB1/TAB2 or TAK1/TAB1/TAB3.

12. Summary of Dissertation Work:

The study presented in this dissertation examines the signaling pathway mediating activin induction of the FSHβ gene, and a method developed for isolating primary gonadotropes. Chapter 1 describes the identification of a Smad-independent pathway mediated by TAK1 MAPKKK. Activin stimulates FSHβ gene expression in the absence of Smad3 as shown by dominant negative inhibitors. We reported that inhibition of TAK1 activation using a dominant negative inhibitor and a chemical inhibitors prevented activin induction of FSHβ gene. These data clearly show that activin stimulation of FSHβ gene is mediated by MAPK signaling cascade initiated by activation of TAK1 and leading to downstream activation of p38-MAPK. Chapter 2 reports the method developed in our laboratory for rapid and efficient isolation of primary gonadotropes from mouse pituitary tissue. The study shows that gonadotropes produce autocrine factors that stimulate FSHβ expression. However, paracrine factors from other cell types play an essential role in robust stimulation of FSHβ gene.

REFERENCES

1. **Sherwood L** 2003 Human Physiology: From Cells to Systems, Fifth ed. Brooks Cole, New York
2. **Kumar TR, Wang Y, Lu N, Matzuk MM** 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201-4
3. **Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi IT** 1997 Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. Nat Genet 15:205-6
4. **Padmanabhan V, Sharma TP** 2001 Neuroendocrine vs. paracrine control of follicle-stimulating hormone. Arch Med Res 32:533-43
5. **Pierce JG, Parsons TF** 1981 Glycoprotein hormones: structure and function. Annu Rev Biochem 50:465-95
6. **Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J** 1996 Gonadotropin-releasing hormone regulates follicle-stimulating hormone-beta gene expression through an activin/follistatin autocrine or paracrine loop. Endocrinology 137:3667-73
7. **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 125:917-24
8. **Haisenleder DJ, Ortolano GA, Dalkin AC, Paul SJ, Chin WW, Marshall JC** 1989 Gonadotrophin-releasing hormone regulation of gonadotrophin subunit gene expression: studies in tri-iodothyronine-suppressed rats. J Endocrinol 122:117-25
9. **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 127:2869-75
10. **Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW** 2004 Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Mol Cell Endocrinol 225:29-36
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 3:1969-76
12. **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 rat anterior pituitary cultures. Endocrinology 128:1682-4
13. **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 142:2275-83
14. **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 132:431-43
15. **Otsuka F, Shimasaki S** 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 143:4938-41
16. **Bishop W, Fawcett CP, Krulich L, McCann SM** 1972 Acute and chronic effects of hypothalamic lesions on the release of FSH, LH and prolactin in intact and castrated rats. Endocrinology 91:643-56
17. **Kawakami M, Higuchi T** 1979 Effects of active and passive immunization with LH-RH on gonadotrophin secretion and reproductive function in female rats. Acta Endocrinol (Copenh) 91:616-28
18. **DePaolo LV, Steger RW** 1985 Alterations in immunoreactive somatostatin levels in hypothalamic and gastroenteropancreatic tissue as a consequence of neonatal treatment with monosodium glutamate. Proc Soc Exp Biol Med 178:419-25
19. **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 U S A 82:7217-21
20. **Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R** 1986 A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem Biophys Res Commun 138:1129-37
21. **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 321:776-9
22. **Attisano L, Wrana JL, Montalvo E, Massague J** 1996 Activation of signalling by the activin receptor complex. Mol Cell Biol 16:1066-73
23. **DePaolo LV, Bicsak TA, Erickson GF, Shimasaki S, Ling N** 1991 Follistatin and activin: a potential intrinsic regulatory system within diverse tissues. Proc Soc Exp Biol Med 198:500-12
24. **Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL** 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. Endocrinology 142:2260-6
25. **Huang HJ, Sebastian J, Strahl BD, Wu JC, Miller WL** 2001 Transcriptional regulation of the ovine follicle-stimulating hormone-beta gene by activin and gonadotropin-releasing hormone (GnRH): involvement of two proximal activator protein-1 sites for GnRH stimulation. Endocrinology 142:2267-74
26. **Pelletier G, Leclerc R, Labrie F** 1976 Identification of gonadotropic cells in the human pituitary by immunoperoxidase technique. Mol Cell Endocrinol 6:123-8
27. **Alarid ET, Windle JJ, Whyte DB, Mellon PL** 1996 Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development 122:3319-29
28. **Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL** 2001 Cell-specific transcriptional regulation of follicle-stimulating hormone-beta by activin and gonadotropin-releasing hormone in the LbetaT2 pituitary gonadotrope cell model. Endocrinology 142:2284-95

29. **Graham KE, Nusser KD, Low MJ** 1999 LbetaT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to active A. J Endocrinol 162:R1-5
30. **Bedecarrats GY, O'Neill FH, Norwitz ER, Kaiser UB, Teixeira J** 2003 Regulation of gonadotropin gene expression by Mullerian inhibiting substance. Proc Natl Acad Sci U S A 100:9348-53
31. **Kumar TR, Low MJ** 1993 Gonadal steroid hormone regulation of human and mouse follicle stimulating hormone beta-subunit gene expression in vivo. Mol Endocrinol 7:898-906
32. **Kumar TR, Fairchild-Huntress V, Low MJ** 1992 Gonadotrope-specific expression of the human follicle-stimulating hormone beta-subunit gene in pituitaries of transgenic mice. Mol Endocrinol 6:81-90
33. **Weiss J, Guendner MJ, Halvorson LM, Jameson JL** 1995 Transcriptional activation of the follicle-stimulating hormone beta-subunit gene by activin. Endocrinology 136:1885-91
34. **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 145:5832-9
35. **Chin D, Boyle GM, Parsons PG, Coman WB** 2004 What is transforming growth factor-beta (TGF-beta)? Br J Plast Surg 57:215-21
36. **Luisi S, Florio P, Reis FM, Petraglia F** 2001 Expression and secretion of activin A: possible physiological and clinical implications. Eur J Endocrinol 145:225-36
37. **Massague J** 1998 TGF-beta signal transduction. Annu Rev Biochem 67:753-91
38. **Ethier JF, Findlay JK** 2001 Roles of activin and its signal transduction mechanisms in reproductive tissues. Reproduction 121:667-75
39. **Thompson TB, Cook RW, Chapman SC, Jardetzky TS, Woodruff TK** 2004 Beta A versus beta B: is it merely a matter of expression? Mol Cell Endocrinol 225:9-17
40. **Carroll RS, Kowash PM, Lofgren JA, Schwall RH, Chin WW** 1991 In vivo regulation of FSH synthesis by inhibin and activin. Endocrinology 129:3299-304
41. **Bernard DJ** 2004 Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells. Mol Endocrinol 18:606-23
42. **Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, Vale W, Sawchenko P** 1989 Production and regulation of inhibin subunits in pituitary gonadotropes. Endocrinology 124:552-4
43. **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 130:1741-3
44. **Derynck R, Zhang YE** 2003 Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577-84
45. **Itoh S, Itoh F, Goumans MJ, Ten Dijke P** 2000 Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem 267:6954-67

46. **Massague J, Wotton D** 2000 Transcriptional control by the TGF-beta/Smad signaling system. Embo J 19:1745-54
47. **Moustakas A, Souchelnytskyi S, Heldin CH** 2001 Smad regulation in TGF-beta signal transduction. J Cell Sci 114:4359-69
48. **Attisano L, Wrana JL** 2002 Signal transduction by the TGF-beta superfamily. Science 296:1646-7
49. **Attisano L, Wrana JL** 2000 Smads as transcriptional co-modulators. Curr Opin Cell Biol 12:235-43
50. **Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT, Wang XF** 1997 Tumor suppressor Smad4 is a transforming growth factor beta-inducible DNA binding protein. Mol Cell Biol 17:7019-28
51. **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-beta signals. FEBS Lett 430:201-4
52. **Safwat N, Ninomiya-Tsuji J, Gore AJ, Miller WL** 2005 Transforming growth factor beta-activated kinase 1 is a key mediator of ovine follicle-stimulating hormone beta-subunit expression. Endocrinology 146:4814-24
53. **ten Dijke P, Hill CS** 2004 New insights into TGF-beta-Smad signalling. Trends Biochem Sci 29:265-73
54. **Luo K** 2004 Ski and SnoN: negative regulators of TGF-beta signaling. Curr Opin Genet Dev 14:65-70
55. **Suszko MI, Lo DJ, Suh H, Camper SA, Woodruff TK** 2003 Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. Mol Endocrinol 17:318-32
56. **Bailey JS, Rave-Harel N, McGillivray SM, Coss D, Mellon PL** 2004 Activin regulation of the follicle-stimulating hormone beta-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol Endocrinol 18:1158-70
57. **Coss D, Thackray VG, Deng CX, Mellon PL** 2005 Activin regulates luteinizing hormone beta-subunit gene expression through Smad-binding and homeobox elements. Mol Endocrinol 19:2610-23
58. **Shibuya H, Iwata H, Masuyama N, Gotoh Y, Yamaguchi K, Irie K, Matsumoto K, Nishida E, Ueno N** 1998 Role of TAK1 and TAB1 in BMP signaling in early Xenopus development. Embo J 17:1019-28
59. **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-beta signal transduction. Science 270:2008-11
60. **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 278:21693-700
61. **Kumar TR, Varani S, Wreford NG, Telfer NM, de Kretser DM, Matzuk MM** 2001 Male reproductive phenotypes in double mutant mice lacking both FSHbeta and activin receptor IIA. Endocrinology 142:3512-8

62. **Terada Y, Nakashima O, Inoshita S, Kuwahara M, Sasaki S, Marumo F** 1999 Mitogen-activated protein kinase cascade and transcription factors: the opposite role of MKK3/6-p38K and MKK1-MAPK. Nephrol Dial Transplant 14 Suppl 1:45-7
63. **Suszko MI, Balkin DM, Chen Y, Woodruff TK** 2005 Smad3 mediates activin-induced transcription of follicle-stimulating hormone beta-subunit gene. Mol Endocrinol 19:1849-58
64. **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-beta signal transduction. Science 272:1179-82
65. **Ono K, Ohtomo T, Ninomiya-Tsuji J, Tsuchiya M** 2003 A dominant negative TAK1 inhibits cellular fibrotic responses induced by TGF-beta. Biochem Biophys Res Commun 307:332-7
66. **Ono K, Ohtomo T, Sato S, Sugamata Y, Suzuki M, Hisamoto N, Ninomiya-Tsuji J, Tsuchiya M, Matsumoto K** 2001 An evolutionarily conserved motif in the TAB1 C-terminal region is necessary for interaction with and activation of TAK1 MAPKKK. J Biol Chem 276:24396-400
67. **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-kappaB signaling pathways. Biochem Biophys Res Commun 297:1277-81
68. **Cheung PC, Nebreda AR, Cohen P** 2004 TAB3, a new binding partner of the protein kinase TAK1. Biochem J 378:27-34
69. **Takaesu G, Kishida S, Hiyama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K** 2000 TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol Cell 5:649-58
70. **Kishimoto K, Matsumoto K, Ninomiya-Tsuji J** 2000 TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop. J Biol Chem 275:7359-64
71. **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 278:18485-90
72. **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-kappaB-activating protein, TAB3. Proc Natl Acad Sci U S A 101:2028-33
73. **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 22:6277-88

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

Nedal Safwat, Jun Ninomiya-Tsuji, A. Jesse Gore, and William L. Miller

Department of Molecular and Structural Biochemistry (N.S., A.J.G., W.L.M.), North Carolina State University, Raleigh, North Carolina 27695-7622; and Department of Toxicology (J.N.-T.), North Carolina State University, Raleigh, North Carolina 27695-7633

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).

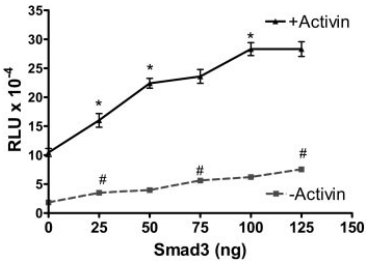


FIG. 1. Overexpression of Smad3 equally stimulated basal and activin-induced expression of oFSHβLuc. LβT2 cells were plated at 25,000 cells/well in 96-well tissue culture plates. After 24 h, they were cotransfected with 50 ng oFSHβLuc and increasing amounts of Smad3 expression construct (25, 50, 75, 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

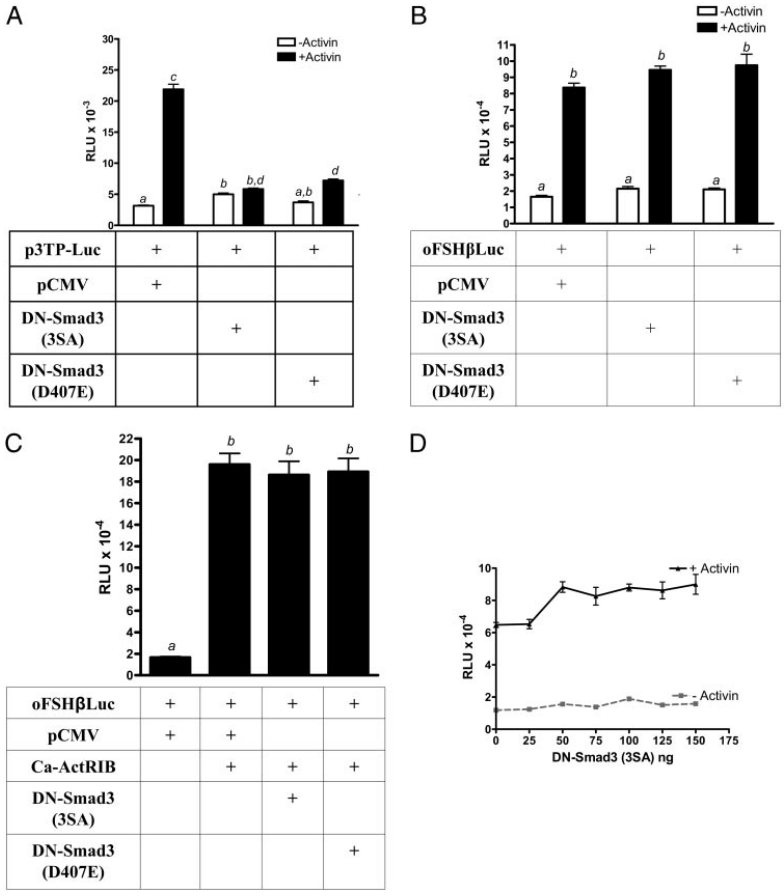


FIG. 2. Activin did not require activated Smad3 to induce oFSHβLuc expression. LβT2 cells were prepared and plated as in Fig. 1 and then treated as follows: A, Cells were cotransfected with 50 ng p3TPLux plus 50 ng pCMV, DN-Smad3 (3SA), or DN-Smad3 (D407E). After transfection (24 h), cells were treated 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

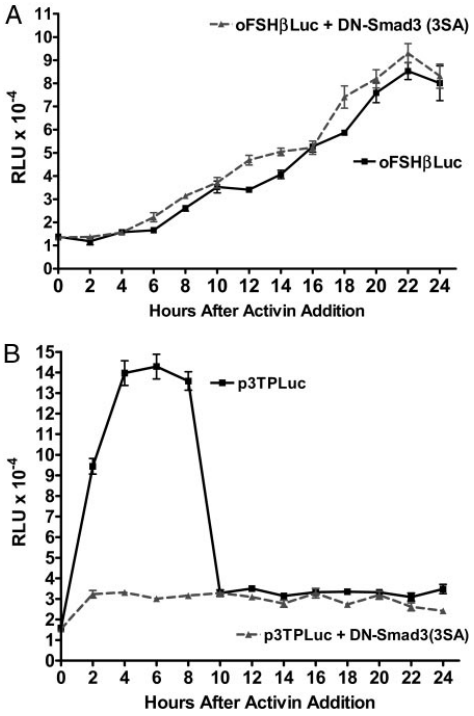


FIG. 3. Different kinetics for oFSHβLuc and p3TPLux induction by activin. LβT2 cells were prepared and plated as in Fig. 1. A, Cells were cotransfected with 50 ng oFSHβLuc plus either 50 ng pCMV (■) or 50 ng Smad3 (3SA) (▲). After transfection (24 h), triplicate cultures were sequentially treated with 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

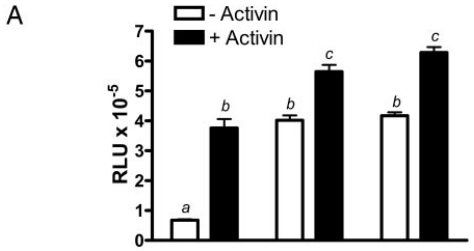
To confirm that 5Z-7-Oxozeanol was a specific inhibitor of oFSHβLuc, the effects of 5Z-7-Oxozeanol were tested on endogenous FSHβ and the two other closely related gonadotropin subunit genes expressed in LβT2 cells. LβT2 cells were treated with activin, with or without 5Z-7-Oxozeanol, and total RNA was 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

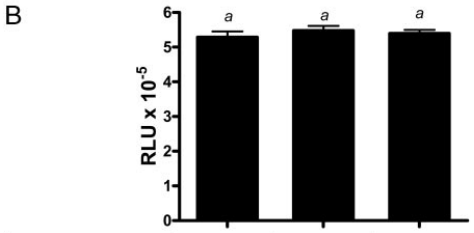
To establish the existence of TAK1 in LβT2 cells and its time-course of phosphorylation by activin, LβT2 cells were pretreated with follistatin for 16 h to bioneutralize any endogenously made activin and then treated with activin for 2–24 h. Cell extracts were prepared and analyzed by Western blot techniques 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

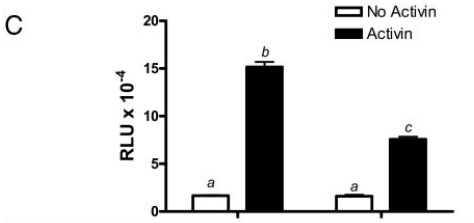
TAK1 is a member of the MAPKKK family that can phosphorylate and activate members of the MAPK family, including the ERK, JNK, and p38 MAPKs. To determine whether MAPKs are involved in activin-mediated induction of oFSHβ, cultures of LβT2 cells were treated with or without specific chemical inhibitors 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)

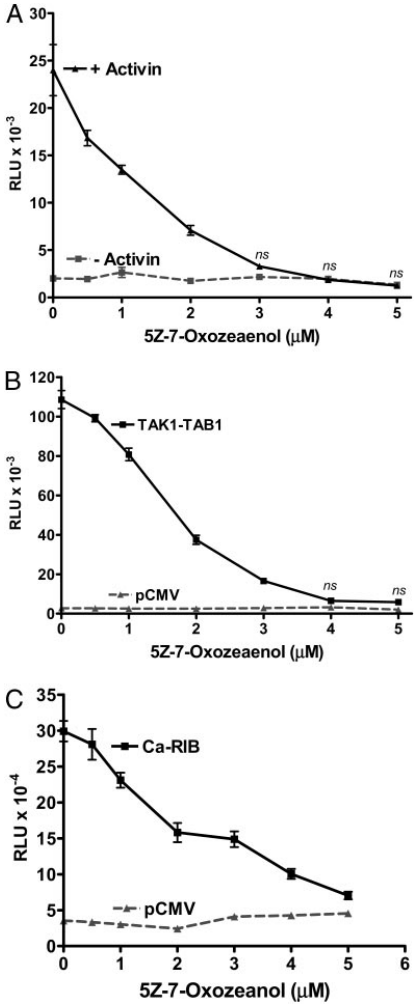


FIG. 5. Inhibition of TAK1 with 5Z-7-Oxozeanol fully blocked activin induction of oFSHβLuc expression. LβT2 cells were prepared and plated as in Fig. 1. A, Cells were transiently transfected with 50 ng oFSHβLuc promoter construct for 24 h. Cells were then treated with or without 5Z-7-Oxozeanol at the 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 ($P < 0.05$).

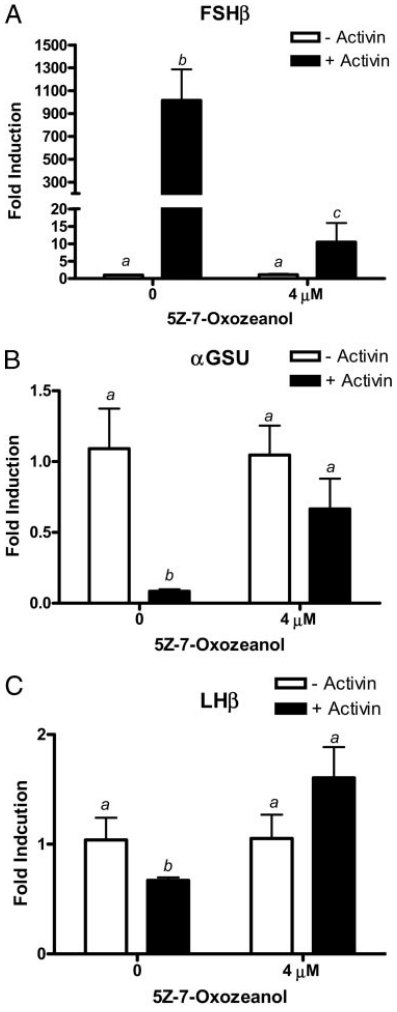


FIG. 6. LβT2 cells were plated in 6-well plates at 1 million cells per well. The cells were treated with or without activin (100 ng/ml) and treated with or without 4 μM 5Z-7-Oxozeanol. Cells were incubated for 24 h and then washed with PBS, and total RNA was extracted using Tri-Zol reagent. Threshold 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 ($P < 0.05$).

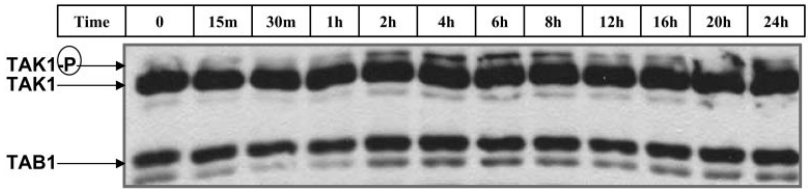


FIG. 7. Activin phosphorylated TAK1 within 2 h and maintained TAK1 activation for 24 h. LβT2 cells were plated at 1 million cells per well in 6-well plates. Cells were pretreated with follistatin-288 (250 ng/ml; 16 h) and washed with culture media. Cells were then treated with activin (100 ng/ml) for 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

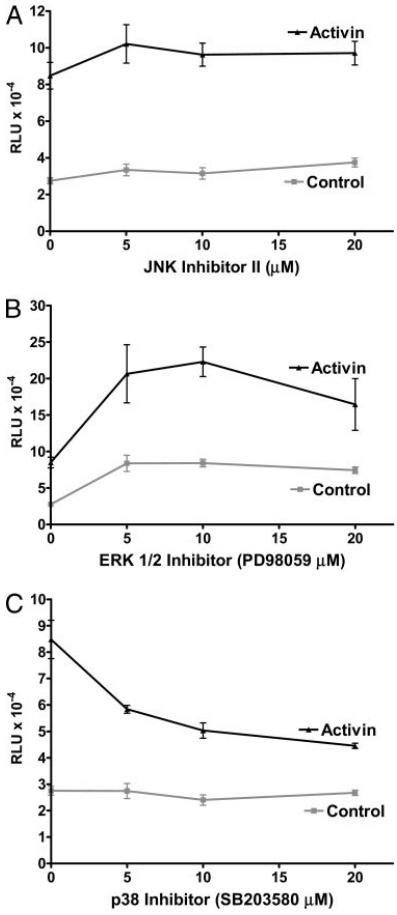


FIG. 8. Inhibition of p38-MAPK partially blocks activin induction of oFSHβ. LβT2 cells were prepared and plated as in Fig. 1A and transiently transfected with 50 ng oFSHβLuc. A, After transfection (24 h), cells were treated with or without activin (100 ng/ml) and with JNK inhibitor II (0, 5, 10, and 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.

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Address all correspondence and requests for reprints to: William L. Miller, Department of Molecular and Structural Biochemistry, Box 7622, North Carolina State University, Raleigh, North Carolina 27695-7622. E-mail: wlmiller@ncsu.edu.

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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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Rapid, Efficient Isolation of Murine Gonadotropes and Their Use in Revealing Control of Follicle-Stimulating Hormone by Paracrine Pituitary Factors

JOYCE C. WU, PEI SU, NEDAL W. SAFWAT, JOSEPH SEBASTIAN, AND WILLIAM L. MILLER

Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-7622

FSH and LH are produced only in gonadotropes, which are reported to comprise 3–12% of mammalian pituitaries. Factors made within the pituitary are powerful regulators of FSH and also influence LH expression, but their identities and cellular origins are unknown because it is impossible to isolate and individually analyze different pituitary cell types. In this study FSH-producing gonadotropes were specifically tagged *in vivo* with a transgenic cell surface antigen (H-2K^k) so they could be purified *in vitro* using paramagnetic anti-H-2K^k-microbeads. After enzymatic dispersion of pituitary cells, it took 1 h or less to extract 55 ± 5% of FSH-producing gonadotropes at 95 ± 0.5% purity, as judged by immunostaining for FSH or prolactin. Although this procedure selected for FSH expression, the isolated gonadotropes were also enriched 22-fold for LH-containing cells. For studies aimed at understanding factors that control FSH transcription, the purified gonadotropes were treated with activin A, which increased FSH expression 480% above basal levels (d 3 of culture). Coincubation of purified gonadotropes with pituitary nongonadotropes increased FSH expression 800% (d 3 of culture). Follistatin, an activin-binding protein, decreased FSH expression 35–50%, suggesting that gonadotropes make some activin and/or other follistatin-sensitive molecule(s) that induce FSH. These data show that paracrine factors from pituitary nongonadotropes can play a major role in controlling FSHβ at the pituitary level. The study presented here describes a rapid, reliable, and efficient method for isolating any specialized cell type, including all cells that produce endocrine hormones. (*Endocrinology* 145: 5832–5839, 2004)

FSH AND LH ARE produced in primary pituitary cell cultures of many species (1, 2) and/or the transformed LβT2 gonadotrope cell line (3, 4). Such synthesis depends on follistatin-sensitive hormones thought to be members of the TGFβ family such as activins (5, 6), bone morphogenetic proteins (BMPs) (7), and follistatin itself (8, 9) made by cells within the pituitary. In fact, most specialized mammalian cells and cell products are partly regulated by paracrine and/or autocrine factors. To understand this type of regulation, it is necessary to separate the specialized cell from its surrounding cells. This is relatively easy if the cell type of interest has unique physical properties, but most specialized mammalian cells do not have unique physical attributes that facilitate separation. Given the plethora of cells that make specific products for critical life functions, it is important to have an approach that can be used broadly to isolate these cells for study. Such a method was devised in this study for isolating gonadotropes, but the method should be applicable to any cell type that produces a unique product.

Gonadotropes, defined by positive immunostaining for LH and/or FSH, are reported to comprise 3–12% of all pituitary cells (10–15), so up to 33-fold purification is required to obtain pure gonadotropin-producing cells. To date, enrichment strategies have worked in the rat, in which most gonadotropes are larger than other pituitary cells. Thus, differential sedimentation at unit gravity (16) was first used for purification and then refined by using greater gravitational force (elutriation) to produce high yields of gonadotropes (1 million gonadotropes from 10 million dispersed rat pituitary cells) that are 95% pure (17). Cells purified by elutriation have been used to study the effects of endocrine and paracrine factors on rat gonadotropes (18), but the procedure is time consuming and has not been extended to other mammals.

Transformed murine gonadotropes (αT3 and LβT2) provide a potential substitute for primary gonadotropes, but these cells are derived from embryonic transformants. α-T3 cells express many proteins specifically associated with gonadotropes (19), but cannot make β-subunits for either of the two mammalian gonadotropins. LβT2 cells were transformed later in development and can produce both gonadotropin β-subunits (20), but like α-T3 cells, they express the simian virus 40 large T antigen, which originally transformed them during embryogenesis and continues to stimulate their cell division. Thus, neither αT3 nor LβT2 cells can be considered fully mature or normal gonadotropes (21, 22).

A novel scheme for purifying gonadotropes recently emerged from work showing that 4.7 kb of the ovine FSHβ promoter specifically express luciferase in gonadotropes (2). In this study (2) the expression of oFSHβLuc was 98 times higher in the pituitary than in any other tissue (activity/mg protein), and it was regulated just like endogenous mouse FSH. In the study reported here, transgenic mice were produced carrying an analogous oFSHβH-2K^k transgene designed to express H-2K^k primarily in FSH-producing gonadotropes. H-2K^k is a major histocompatibility protein absent

Abbreviations: BMP, Bone morphogenetic protein; o, ovine; PRL, prolactin; rtPCR, real-time PCR.

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in most mouse strains used for transgenic work (www.informatics.jax.org) (23). It lacks protease-sensitive sites on its extracellular amino terminus, so it is not digested by enzymes used to disperse mammalian cells. Furthermore, the particular H-2K^k used here lacks an intracellular carboxyl terminus, so it has no intracellular signaling ability to interfere with normal cell functions. Another advantage of using H-2K^k is the recent development of a commercial technique that uses magnetic immuno-microbeads to rapidly and efficiently isolate whole cells that express H-2K^k on their cell surface.

Reported here are the details of isolating primary mouse gonadotropes that actively produce FSH and their initial use to probe the complex processes involved in regulating FSH by follistatin, TGFβ family members, and/or unknown paracrine factors produced in the pituitary.

Materials and Methods

The oFSHβH-2K^k construct

Ovine (o) FSHβH-2K^k was made by substituting the H-2K^k gene for luciferase in oFSHβLuc (2). The H-2K^k cDNA was amplified by PCR from the pMACs Kk.II expression plasmid (2472–3575 bp; Miltenyi Biotec, Inc., Auburn, CA), and *Xba*I and *Nco*I restriction sites were created on the 5′ and 3′ ends of this gene, respectively, using the PCR oligonucleotide primers: 5′-cggtaccccacTCTAGAaaccaacacac-3′ (*Xba*I site in *capital letters*) and 5′-cgcCCATGGcgatggcaccctgcatgc-3′ (*Nco*I site in *capital letters*). The PCR product was ligated into pCR-Blunt II-TOPO vector (Invitrogen Life Technologies, Inc., Carlsbad, CA), cut from it with *Xba*I and *Nco*I restriction enzymes, and then ligated into a pGL3 vector to replace the luciferase gene that was cut out using *Xba*I and *Nco*I restriction enzymes (Promega Corp., Madison, WI). Finally, the pGL3-H-2K^k construct was opened at its polylinker with *Kpn*I and *Sma*I restriction enzymes, and the 4.7-kb promoter plus the first intron of the oFSH β-subunit was directionally ligated into this site, as previously reported for oFSHβLuc (2). The H-2K^k cDNA does not encode the carboxyl terminus of H-2K^k involved in signal transduction, but does contain 326 amino acids of the amino terminus, which extend from the cell surface (Miltenyi Biotec). Finally, there are no tryptic cleavage sites in the H-2K^k amino terminus, and we experimentally showed that treatment with either collagenase (type I; Sigma-Aldrich Corp., St. Louis, MO) or pancreatin (porcine pancrease; Sigma-Aldrich Corp.) did not destroy the ability of H-2K^k to act as an effective cell surface antigenic hook (unpublished observations of Dr. J. Sebastian, our laboratory).

Generation and screening of transgenic mice

The oFSHβH-2K^k fragment was linearized by digestion with *Bam*HI and *Kpn*I restriction nucleases, purified, and microinjected into the pronuclei of fertilized B6SJL mouse eggs as described previously (2). Genomic DNA was purified from mouse tails as reported and was tested for the presence of oFSHβH-2K^k using PCR to create a diagnostic fragment 339 bp long using the following primers specific for H-2K^k: 5′-caatagtcactggagctgtggtggc-3′ and 5′-ctcccacacctccccctgaacctgaaac-3′. Sixteen founder mice were obtained and mated with CD-1 mice (Charles River Laboratories, Raleigh, NC). Only two of the best founder lines were kept and bred to each other to obtain homozygous oFSHβH-2K^k mice or to CD-1 mice to produce large numbers of hemizygous oFSHβH-2K^k mice.

Mice carrying two transgenes (oFSHβH-2K^k and oFSHβLuc) for the expression of both H-2K^k and luciferase in their gonadotropes were obtained by breeding homozygous oFSHβH-2K^k mice with homozygous oFSHβLuc mice (founder line 7913 in Ref. 24).

Finally, mice were housed in the Biological Resource Facility of North Carolina State University. Gonadotropes that were isolated from the pituitaries of ovariectomized females were isolated 2 wk after ovariectomy. All mice were cared for according to the rules and regulations of the institutional animal care and use committee of North Carolina State University.

Purification of gonadotropes

Pituitaries from two to 21 mice were dispersed using collagenase and pancreatin as previously reported (2) and were filtered through a 27-μm pore size nylon mesh to remove undigested tissue. Cells were then collected by centrifugation (155 $\times$ *g*) and suspended in 100 μl freshly degassed PBS buffer (complete degassing is absolutely necessary) containing 4 mM EDTA, 0.5% BSA, and 10 μl biotin antimouse H-2K^k (BD Pharmingen, San Diego, CA). Cells were gently rotated for 10 min at 4 C, and then 2 ml PBS (EDTA/BSA) buffer were added before the cells were centrifuged again at 155 $\times$ *g*. Cells were incubated with 100 μl PBS (EDTA/BSA) buffer plus 20 μl antibiotin paramagnetic microbeads (Miltenyi Biotec), rotated for 15 min at 4 C, washed with 2 ml buffer as before, and suspended in 1 ml PBS (EDTA/BSA) buffer. The cells were added to and eluted from a 0.2 $\times$ 2-cm magnetic separation column (high gradient MS$^+$, Miltenyi Biotec) that had been prewashed with 0.5 ml buffer according to instructions by Miltenyi Biotec. Cells flowing directly through the column while it was in the magnetic field were analyzed as flow-through cells (not magnetically attracted to the column). Cells attracted to the column were subsequently eluted after removal of the magnetic field and were analyzed as enriched gonadotropes having undergone one cycle of purification (see Fig. 1B). Cell counts for all dispersed cells were performed in triplicate using a hemocytometer. For the studies summarized in Figs. 2 and 3, cells were enriched to 95%

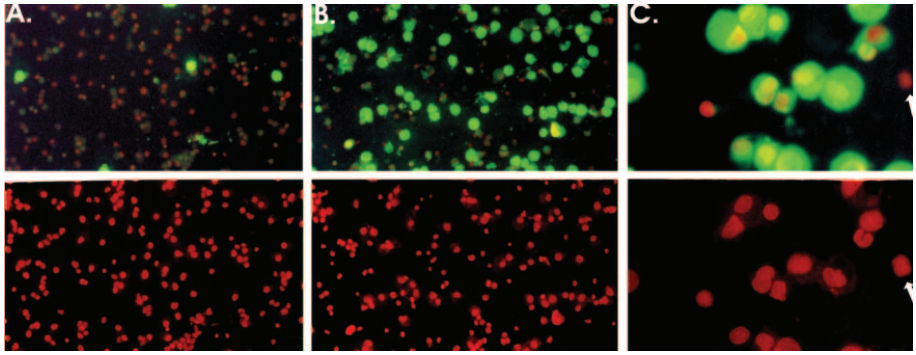

FIG. 1. Gonadotrope purification from a representative preparation of dispersed pituitary cells. A, Some 3.2% ± 0.2% of freshly dispersed cells stained for FSH (*top row* shows FSH, *green*; *bottom row* shows nuclei, *red/orange*; magnification, ×125). B, About the same number of cells was analyzed as shown in A, but almost half the cells stained green for FSH (*top row*) after one cycle of enrichment (magnification, ×125). C, Highly enriched gonadotropes after two cycles of purification (magnification, ×500). Note that gonadotropes are large, medium, and small in size.

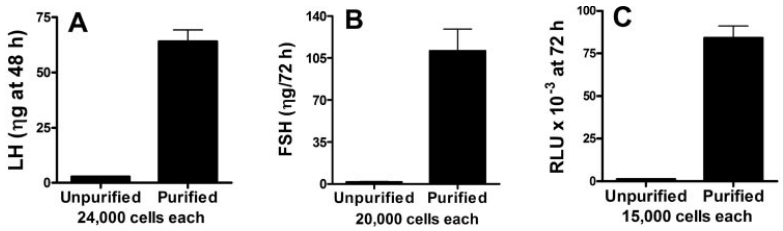

FIG. 2. Expression of LH, FSH, and luciferase in unpurified cell preparations and purified gonadotrope fractions. A, Some 24,000 dispersed unpurified pituitary cells or purified gonadotropes were cultured in triplicate for 2 d, and the media were assayed for LH by RIA. B, Twenty thousand dispersed unpurified pituitary cells or purified gonadotropes were cultured in triplicate for 3 d, and the media were assayed for FSH by RIA. C, Fifteen thousand freshly dispersed unpurified pituitary cells or purified gonadotropes were cultured in triplicate for 3 d, and the cells were assayed for luciferase activity. Pituitaries in C were obtained from mice harboring the oFSHβLuc transgene.

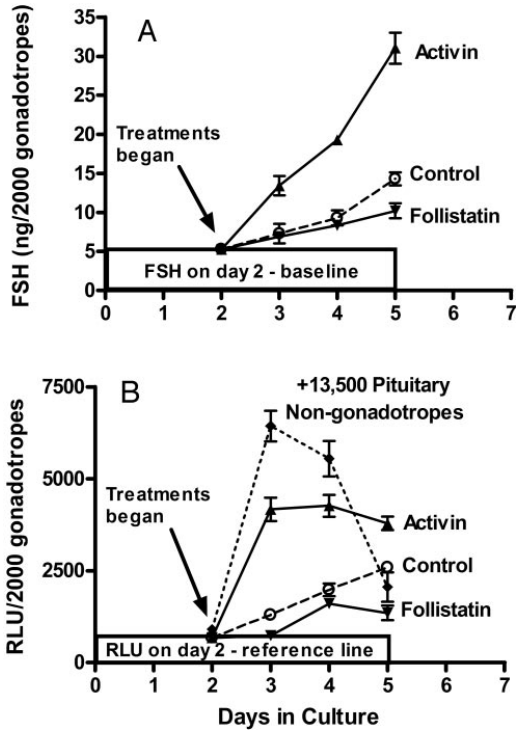

FIG. 3. Expression of FSHβ in 95% pure gonadotropes exposed to gonadotrope-depleted pituitary cells, follistatin, or activin. Purified gonadotropes (n = 2,000) were incubated in 384-multiwell culture plates for 2, 3, 4, or 5 d before analysis. Cultures were divided into the following groups on d 2: A, follistatin-treated (100 ng/ml); B, no treatment; and C, activin-treated (100 ng/ml). A fourth group, started on d 0, involved coincubation of 2,000 purified gonadotropes with 13,500 dispersed pituitary cells from the flow-through fraction of magnetic bead separation, which were significantly depleted of gonadotropes (labeled pituitary non-gonadotropes). Data from this fourth group are shown only in B. Because culture medium was not changed after cell plating, the FSH results (A) represent accumulated hormone from d 0. The data in B indicate luciferase activities [as relative light units (RLU)] associated with each culture well. Luciferase activities and FSH values were from the same cultures, and the data represent the mean ± SEM of three separate experiments, each performed in triplicate.

purity using two consecutive cycles through two separate MS^+ columns (see Fig. 1C).

Analysis of gonadotrope purity

Dispersed pituitary cells were centrifuged onto polylysine-coated slides (10,000–20,000 cells/slide) using a Cytospin centrifuge (Shandon Southern, Pittsburgh, PA) at 1,000 rpm for 10 min, fixed for 1 h with freshly prepared 2% paraformaldehyde, washed three times with PBS (5 min each), and then stored in PBS until use. Cells were immunostained for FSH, LH, prolactin (PRL), or GH and chemically stained for nuclei. The nuclear stain (Vectashield with propidium iodide, Vector Laboratories, Inc., Burlingame, CA) was applied in the coverslip mounting solution to permit an accurate cell count on all slides (see Fig. 1, *red/orange* nuclear stain on the *bottom row*; the stain was strong enough to be visualized in the *top row* also). Rabbit antimouse polyclonal antibodies for FSH (H1426) or LH (H5346) were used at a 1:200 dilution for 30 min at 37 C to specifically label mouse FSH or LH (Accurate Chemical & Scientific Corp., Westbury, NY; no. A581/RH4), followed by a 30-min incubation at 25 C with a 1:40 dilution of fluorescein isothiocyanate-labeled goat antirabbit antibody (H+L) as second antibody (Zymed Laboratories, San Francisco, CA). Cells containing PRL or GH were identified using first antibodies from the NIDDK (PRL, AFP-131078; GH, AFP5641801) at dilutions of 1:20 and 1:200, respectively, followed by the second antibody noted above. Before incubation with either the first or second antibody, cells were washed with PBS and incubated for 20 min with blocking solution (10% charcoal-treated sheep serum plus 10% brain-heart infusion, BD Biosciences, Cockeysville, MD).

Gonadotropes in culture

Purified gonadotropes and/or flow-through cells were cultured in medium 199 (Invitrogen Life Technologies, Inc., Gaithersburg, MD) with 10% charcoal-treated sheep serum and antibiotics/antimycotics as previously reported (2). The data in Fig. 2 were obtained from 15,000–24,000 cells cultured in 100–200 μl medium in 96-well Primaria culture plates (BD Biosciences, Franklin Lakes, NJ). The data in Fig. 3 were obtained by incubating 2,000 cells in 80 μl medium using 384 well plates coated with poly-D-lysine (781940P) from Greiner Bio-One (Longwood, FL).

RIA for FSH and LH

The levels of FSH and LH in culture medium were measured with reagents provided by the National Pituitary and Hormone Program of the NIDDK using a double antibody method previously described (1, 2). All samples were assayed in duplicate from each medium sample obtained from triplicate culture wells; the intraassay variation was 8% or less. Culture media were collected and frozen at −20 C before RIA. For the FSH RIA, rabbit anti-oFSH antiserum (AFP-C5288113) was used as the first antibody, rat FSH (AFP-11454B) was used as iodinated tracer, and mouse FSH (AFP-5308D) was used as the reference protein. For the LH RIA, rabbit antirat LH antiserum (AFPC697071P) was used as the first antibody, rat LH (AFP-115368) was used as iodinated tracer, and mouse LH (AFP-5306A) was used as the reference preparation. The

second antibody was sheep antirabbit antiserum prepared in our laboratory and used as previously reported (1, 2).

Real-time RT-PCR (RT-rtPCR)

Total mouse RNA was isolated using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to Tri-Reagent instructions. Triplicate wells of a 96-well culture plate were plated with 6000 purified gonadotropes and treated in the same way as cells shown in Fig. 3 for up to 3 d. Then media were removed, and cells were treated with 0.8 ml Tri-Reagent along with 4 μl Polacryl Carrier (Molecular Research Center). Total RNA was converted to cDNA using the iScript cDNA Synthesis kit from Bio-Rad Laboratories (Hercules, CA). The PCR probes for mouse FSHβ cDNA were 5′-AGAGAAGGAAGAGTGCCGTTTCTG-3′ (forward) and 5′-ACATACTTTCTGGGTATTGGGCCG-3′ (reverse), and the TaqMan probe was (6-carboxy fluorescein) 5′-ATCAATACCACTTGGTGTGCGGGCTA-3′. The internal standard was mouse 18S ribosomal RNA, which was measured as cDNA using the following oligonucleotides: 5′-GAAACTGCGAATGGCTCATTAA-3′ (forward; 966–987 bp), 5′-GAATCACCACAGTTATCCAAGTAGGA (reverse; 1046–1021 bp), and (6-carboxy fluorescein) 5′-ATGGTTCCTTTGGTCGCTCGCTCC-3′ (995–1018 bp). rtPCR was performed according to Bio-Rad Laboratories in the iCycler, and values, relative to the control, were calculated using the $2^{-\Delta\Delta Ct}$ method (25).

Luciferase assay

Luciferase activity was quantified on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) using the Promega luciferase assay system as previously reported (2).

Statistics

Statistical calculations were performed using PRISM (version 4, GraphPad, Inc., San Diego, CA). The mean ± SEM in Fig. 1 were obtained by counting 1000 cells or more from each of three separate dispersed cell preparations. The data in Fig. 2, A–C, were obtained from three separate gonadotrope isolations. Significant differences between means were calculated using one-way ANOVA, followed by Tukey's multiple comparison testing for Fig. 1 and rtPCR data associated with Fig. 3. Differences in Fig. 1, A–C, gonadotrope purifications were significant, with $P < 0.05$. A *t* test was used for analyzing the data in Fig. 2, where differences between unpurified cells and purified gonadotropes had a $P < 0.001$ in A–C.

Results

Four transgenic founder lines were shown to support significant gonadotrope purification, and one line (H-2K^k-7) was chosen for all of the studies reported here because it provided the best purification and was the most prolific. A second line gave similar purification results (H-2K^k-5). Studies with these two founder lines showed that gonadotropes were efficiently purified only from the pituitaries of males or ovariectomized females. Careful attention to cycle timing for intact females might find suitable gonadotrope purification associated with estrus, but this type of experiment has not yet been performed.

Figure 1 shows sequential pictures for the purification of dispersed male pituitary cells. All cells were stained for both FSH (*green*) and nuclei (*red/orange*). Figure 1A shows cells that were dispersed and prepared for purification, but did not go through a magnetic bead column. The percentage of gonadotropes in these unpurified cells was 3.2 ± 0.2%. This percentage is not significantly different from that found in CD-1 mice treated the same way (2.6 ± 0.1%; n = 3), showing that the expression of H-2K^k in gonadotropes had no significant effect on the number of gonadotropes in dispersed mouse pituitary preparations.

Figure 1B shows that after one cycle of purification, gonadotropes comprised 47 ± 3.5% of the cells. The percentage of gonadotropes in the flow-through fraction from the first cycle of column purification was 0.7 ± 0.06. Comparison with the 3.2 ± 0.2% population of gonadotropes entering the column indicated a 22% loss (78% recovery) of gonadotropes due to cells not binding to the column. The second column showed a flow-through loss of 16 ± 3% (84% recovery), giving a cumulative loss of 38 ± 5% for both purification cycles, which is not statistically different from the observed loss of 45 ± 5% (55 ± 5% recovery). Finally, Fig. 1C shows gonadotropes that underwent two cycles of enrichment. They were considered approximately 95% pure based on immunostaining for FSH.

The purity of gonadotropes after two cycles of isolation was difficult to quantify because small numbers of nuclei were present that had lost their cyctoplasm, and their original cell type was undefined (see *white arrows* in Fig. 1C). Also, occasional cells contaminated the gonadotrope fraction, but two to four cells would make a large difference in calculating purity. To obtain an independent estimate of gonadotrope purity, lactotropes were stained and counted in the original mixed cell preparation and in two-cycle enriched gonadotrope fractions. Lactotropes are small, durable, and easily stained cells. Furthermore, PRL has never been reported to coexist with FSH in male gonadotropes. The percentage of lactotropes in the unpurified dispersed pituitary cells varied from 25.6% to 35.6% in three separate preparations (mean, 31 ± 4%), but the level of lactotropes consistently dropped by 95% in each purified gonadotrope preparation (*i.e.* there was an average of only 1.5% lactotropes in highly purified gonadotrope fractions; data not shown). Based on the difference in lactotrope abundance in the unpurified and two-cycle enriched gonadotropes, it was calculated that the purified gonadotrope fraction contained 95.3 ± 0.5% gonadotropes.

The data in Table 1 show the number of purified gonadotropes obtained from four separate preparations of dispersed male pituitaries (two to 21 pituitaries/preparation). The average number of total dispersed pituitary cells ob-

TABLE 1. Yields of dispersed mouse pituitary cells and purified gonadotropes

Pituitaries	Dispersed cells total	FSH cells total[a]	Yield of FSH cells	% Yield of FSH cells
2	1,000,000	32,000	22,000	69
20	10,000,000	320,000	150,000	47
21	13,000,000	390,000	200,000	48
18	7,200,000	130,000	130,000	56
Average/pituitary	$0.5 \pm 0.04 \times 10^6$	16,000	8,800 ± 900	55 ± 5

[a] Calculation based on data in Fig. 1, showing that 3.2% of dispersed cells contain FSH.

tained per mouse was 0.5 ± 0.04 million, and the average number of purified gonadotropes was 8800 ± 900/pituitary. Assuming that gonadotropes represented 3.2% of each preparation (see Fig. 1A), the overall recovery of enriched gonadotropes was estimated to be 55 ± 5% after two cycles of purification.

The purified gonadotropes (95% FSH-staining cells) were also stained for mouse LH to quantify cells containing FSH/LH or FSH only (Fig. 2A). In contrast to the intense and uniform immunostaining observed for mouse FSH (Fig. 1), LH immunostaining varied widely, from very intense to barely visible above background, which made accurate quantitation by immunostaining impossible (data not shown). As an alternative to immunostaining, RIA was used to quantify LH in the media of an equal number of purified and unpurified gonadotropes (24,000 cells/well; see Fig. 2A). The data in Fig. 2A indicate that purified gonadotropes contained 22 times more LH per cell than unpurified cells (64 ± 5.2 *vs.* 2.8 ± 0.2), indicating a 22-fold enrichment of LH-containing cells in the gonadotrope fraction.

In Fig. 2B, mouse FSH was quantified by RIA in cultures of unpurified and purified gonadotropes (20,000 cells/well) that were cultured for 3 d in a 96-well plate. The FSH produced by 20,000 purified gonadotropes was 75 times greater than that produced by 20,000 unpurified cells cultured under identical conditions (111 ± 18 and 1.5 ± 0.6 ng/well, respectively). These results are from one experiment, but are typical of three separate experiments.

In a third experiment, 15,000 purified gonadotropes were cultured as described above, and the expression of the oFSHβLuc transgene was measured. In this study luciferase activity was 74 times greater in cultures of purified gonadotropes than in unpurified cells (Fig. 2C). That is, highly enriched gonadotropes expressed $84 \pm 7 \times 10^3$ relative light units, whereas unpurified cells expressed only $1.1 \pm 0.06 \times 10^3$ relative light units. The data in Fig. 2, B and C, show that the expression of both endogenous mouse FSH and oFSHβLuc copurified to the same extent in the enriched gonadotropes.

The effects of follistatin, activin, and unidentified pituitary paracrine factors were tested on FSH expression in purified gonadotropes (Fig. 3). Double-purified gonadotropes that expressed oFSHβLuc along with endogenous mouse FSH were incubated for 5 d in 384-well culture plates (2000 cells/well). Untreated cultures expressed relatively high levels of FSH (Fig. 3A) and luciferase (Fig. 3B) in the absence of any added hormones. Addition of follistatin on d 2 partially decreased both FSH secretion and luciferase activity by approximately 35% (on d 5). By contrast, activin treatment on d 2 increased the rate of FSH accumulation and luciferase activity by 480% on d 3 compared with those after control or follistatin treatment. The data in Fig. 3, A and B, came from the same cultures, but appear different because FSH is an accumulated value (accumulation of mouse endogenous FSH from d 0), whereas luciferase activity represents steady state luciferase expression at the time of analysis.

To determine the effect of paracrine factors on FSH expression, 13,500 pituitary cells from the flow-through fraction of purification were coincubated with 2,000 purified gonadotropes from d 0 of culture. After correcting for residual luciferase activity in the flow-through fraction, it was found that the 2,000 purified gonadotropes expressed 8 times more oFSHβLuc than cultures treated with follistatin. By d 5 of culture, however, luciferase activity dropped dramatically to a level somewhere between that of control and follistatin-treated cultures (Fig. 3B). These data show that paracrine factors that act like activins can play a dominant role in inducing the expression of FSH, but other factors, such as follistatin or other inhibitory substances, are eventually made by nongonadotropes that cause a precipitous fall in FSHβ expression by d 5 (Fig. 3B).

To confirm that the oFSHβLuc activity shown in Fig. 3B accurately reflected steady state levels of endogenous mouse FSHβ mRNA, rtPCR was used to quantify mouse FSHβ mRNA. Total RNA was isolated from two independent gonadotrope cultures on d 3 that were treated as described in Fig. 3B. Ribosomal subunit 18S was used as the internal control, which did not change with treatment and had an average threshold cycle value of about 15. Based on changes in threshold cycle values for mouse FSHβ mRNA (ct), it was determined that activin significantly increased mouse FSHβ mRNA by 5- or 6.9-fold in the two preparations ($P < 0.01$), whereas follistatin decreased mouse FSHβ mRNA by 49% ($P < 0.05$).

Finally, the coexistence of GH and FSH in double-purified gonadotropes was tested by immunostaining highly enriched gonadotropes for GH. Cells that stained for GH in unpurified pituitary cells comprised approximately 39%. Therefore, if gonadotropes were totally devoid of GH, there should have been about 1.9% of GH cells in the highly purified gonadotrope preparation. There were, in fact, 12 ± 2% of highly enriched gonadotropes that stained for GH, indicating that approximately 10% ± 2% of the male mouse gonadotropes contained both FSH and GH.

Discussion

As reviewed in the introduction, it is important to study gonadotrope regulation by paracrine and/or autocrine factors made in the pituitary, but this cannot be done effectively without separating gonadotropes from their surrounding cells. Separating gonadotropes has been very difficult in the past, and this report describes a rapid, efficient, highly reliable, and relatively inexpensive method for isolating primary gonadotropes that may be of considerable use in the future not only for gonadotropes, but for any cell type that expresses a unique gene product. The only criterion that discriminates between cells that are isolated and those left behind (flow-through) is the appearance of H-$2K^k$ on the gonadotrope cell membrane that is proportional to FSHβ expression in the cell. Therefore, this new method selects specifically for gonadotropes that are actively producing FSH. Because LH is often made along with FSH (≥85% of gonadotropes are usually FSH/LH cells), a majority of LH-containing cells would also be expected to be isolated, and there was, in fact, a 22-fold enrichment for LH in the gonadotrope fraction. Some LH-producing cells do not make FSH (5–15% in other species) (10–15), so it is also likely that some LH-producing cells were not isolated in the gonadotrope fraction. Nevertheless, 22-fold enrichment predicts 100%

pure LH cells if LH cells originally comprised just 4.5% of the pituitary cells, suggesting that LH enrichment was quite high in the gonadotrope fraction.

It was surprising to find, however, that the overall percentage of gonadotropes in the male mouse pituitary was only 3.2%, because 10% or more of rat pituitary cells have been identified as gonadotropes (17). The low percentage might result from selective destruction of a majority of mouse gonadotropes during the dispersion process, but this should lead to widely varying percentages of gonadotropes in different mouse pituitary preparations, which was not observed. A more likely reason for low gonadotrope numbers is that a majority of mouse gonadotropes might be prescient gonadotropes that carry GnRH receptors but produce very little FSH or LH and are not easily detected by immunostaining or RIA. Of course, it may be that the mouse can function with fewer gonadotropes than other mammals. Immunostaining and quantitative analysis of whole mouse pituitary tissue will eventually resolve this issue.

The purification procedure described here yielded 95% pure primary FSH-containing gonadotropes at 55 ± 5% yield (~8,800 gonadotropes/mouse pituitary). As noted in *Results*, there was an average loss of 22 ± 2% in the flow-through fraction for each cycle of the two-cycle purification. This accounts entirely for the losses that result in the observed 55% overall yield. During manuscript preparation, the length of collagenase incubation was increased to 2 h from 1.5 h to produce an average of 1,000,000 pituitary cells/mouse, which is a 2-fold increase over that shown in Table 1. Altering column parameters to capture more gonadotropes also has a potential for doubling the yield, which could produce up to 35,000 gonadotropes/mouse pituitary.

The purified gonadotropes were easily cultured and analyzed for FSHβ expression by measuring mouse FSH mRNA (rtPCR), mouse FSH (RIA), or oFSHβLuc activity that reflected expression from 4.7 kb of the ovine FSHβ promoter. As few as 2000 highly enriched gonadotropes were cultured per well in a 384-multiwell culture plate, meaning that 11 mouse pituitaries were required to obtain data from 50 individual singlet treatments. Luciferase activity was particularly useful because it provided a rapid assay that reflected steady state FSHβ mRNA concentrations (measured by real-time rtPCR) at any moment in time and is often used as an estimate for transcriptional activity. An entire luciferase assay (60 wells) takes less than 0.5 h to complete, which makes it 100–200 times faster than either real-time rtPCR or RIA. The FSH RIA measured accumulation of FSH over time and was valuable because it monitored a combination of processes that encompassed transcription, mRNA stability, translation, protein processing, and secretion. Because data from the FSH RIA and rtPCR were similar, it was concluded that follistatin or activin only altered FSHβ mRNA levels and not mRNA translation, protein processing, or secretion.

Because only 2000 cells were required per well in a 384-well plate, a clear advantage of this experimental model is its use of only 30–80 μl culture medium/treatment. This means that the consumption of hormones during treatments is relatively small (activin, follistatin, BMPs, inhibin, GnRH, and others). This is important, because commercially available recombinant follistatin and TGFβ family members are costly. Even more important, the bioassay provided by 95% pure primary gonadotropes may permit the rapid screening of minute quantities of unknown paracrine factors that alter FSH expression.

It is instructive to compare FSH production from transformed LβT2 gonadotropes and the purified primary gonadotropes reported here. Purified primary gonadotropes, without activin treatment, produced 2.8–3.7 ng FSH/d·2000 cells. This rate of FSH production is 1000 times greater than that found for an equivalent number of LβT2 cells in culture without activin (3, 4). Even with activin treatment, LβT2 cells produce only 0.007 times the amount of FSH produced by an equivalent number of primary pituitary gonadotropes treated with activin. One cause of such a large difference might be the rapid rate of LβT2 cell division; perhaps only a small number of LβT2 cells are in G_1 producing FSH at any given time. A second possibility is that LβT2 cells may benefit from factors that primary gonadotropes are exposed to *in vivo* (paracrine or endocrine), which they "remember" in tissue culture. Finally, it is possible that LβT2 cells produce less FSH because the large T antigen interferes with important cellular functions (21, 22). Comparative studies between LβT2 cells and pure primary gonadotropes should help determine which of the above possibilities is correct.

Follistatin inhibits FSH expression by binding and incapacitating activin or other TGFβ family members that may stimulate FSH expression (26, 27) and are sensitive to follistatin. Because FSH is inhibited 35–50% after treatment with follistatin in both purified primary gonadotropes and LβT2 cells (4), it seems likely that both cell types produce significant amounts of either activin- or follistatin-sensitive TGFβ family member(s) as autocrine factors that stimulate FSH expression. It is worth noting in Fig. 3B that purified gonadotropes naturally increase luciferase expression from d 2–5, suggesting that they produce autocrine substances that may or may not be TGFβ family members because follistatin blocks only some of the increase. These data are consistent with studies that have identified activin and/or BMP subunit mRNAs in LβT2 cells, pituitary cultures, or primary gonadotropes (4, 26–28), but the relatively low level of FSH induction compared with FSHβ induction by paracrine factors from nongonadotropes (see discussion below) suggests that these autocrine factors may not be the primary inducers of FSH in the pituitary.

Activin treatment caused a significant increase in FSH expression (480%) as expected, but exposure to pituitary nongonadotropes increased FSH expression even further (800%) compared with expression in the presence of follistatin on d 3 of culture. These data suggest that the expression of FSH is stimulated most dramatically by paracrine factors from nongonadotropes. It is not known whether these factors are TGFβ family members like activin. They may be general growth factors that stimulate gonadotropes to produce TGFβ family members, but the answer to this possibility awaits further study. Interestingly, it is clear in Fig. 3 that basal expression of FSH increased even when follistatin was present, so gonadotropes may produce autocrine stimulators of FSH production that are not TGFβ family members.

Coculture of purified gonadotropes with nongonadotropes on d 5 expressed much less FSH than on d 3. This was

not due to down-regulation by activin action, because constant stimulation by activin alone produced a continuous high level of oFSHβluc expression, but it could have been due to synthesis of follistatin (29) or down-regulation by factors other than activins. It is not yet clear what pituitary-made paracrine and/or autocrine factors are most influential in regulating FSH, but the use of primary gonadotropes to detect these factors has the potential for isolating and identifying the molecules that are so important in regulating FSH, follicular development, and fertility in mammals.

For years it has been known that GH and FSH can coexist in the same pituitary cell in female rats. Furthermore, there is significant evidence showing that a portion of GH cells can cycle in and out of producing gonadotropins and supplement gonadotrope populations when required (30). This indicates significant plasticity in the GH and gonadotrope cell types, which may now be studied in tissue culture in the absence of large numbers of GH-only cells. Because GH expression has not been reported for LβT2 cells, purified primary gonadotropes represent a unique opportunity for studying hormones that control the transitions between somatotropes and gonadotropes. The abundance of GH/FSH-containing cells has not yet been determined for gonadectomized animals or females during estrous; the numbers of these dual-secreting cells may be significantly greater in these populations, which would make it even easier to study the transitions from somatotrope to somato-gonadotrope to gonadotrope.

Finally, the procedures used in this study can be applied to numerous mammalian cell types that express unique proteins. These cell types include the majority of pituitary cells as well as pancreatic, kidney, adrenal, ovary, testicular, mammary, pineal, ocular, cochlear, neural, and immunological cells; all endocrine cells; and many other cell types. Most of these cell types are likely to be heavily influenced by paracrine and/or autocrine hormones, making it essential to study them in isolation from surrounding cells to understand their most critical regulation. The methods presented here can be used to isolate any of these cell types as long as a promoter can be found to express H-2K^k specifically in the cell type of interest. Even the use of luciferase transgenes can be applied to all cell types for studies in many fields of endocrinology once a suitable promoter is identified.

In summary, an inexpensive, rapid, efficient, and reproducible method for isolating 95% pure primary gonadotropes is reported. The method is broadly applicable to all specialized cell types. Production of FSH and expression of FSHβ, as measured by expression of an oFSHβLuc transgene, were easily measured using as few as 2000 purified gonadotropes. Production of FSH in purified primary gonadotropes was induced 1) about 35% by follistatin-sensitive factors thought to be activin-like hormones produced within gonadotropes; 2) 480% by recombinant activin A; or 3) 800% by factors from pituitary nongonadotropes, which might play a dominant role in FSH expression *in vivo*. Finally, about 10% of purified primary gonadotropes contained both GH and FSH. These cells offer a model for studying the factors that interconvert somatotropes and somato-gonadotropes.

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Address all correspondence and requests for reprints to: Dr. William L. Miller, Department of Molecular and Structural Biochemistry, Box 7622, North Carolina State University, Raleigh, North Carolina 27695-7622. E-mail: wlmiller@ncsu.edu.

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Current address for J.S.: Labcorp, Research Triangle Park, North Carolina 27709.

References

1. **Miller WL, Wu JC** 1981 Estrogen regulation of follicle-stimulating hormone production *in vitro*: species variation. Endocrinology 108:673–679
2. **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
3. **Graham KE, Nusser KD, Low MJ** 1999 LβT2 cells secrete follicle stimulating hormone (FSH) in response to activin A. J Endocrinol 162:R1–R5
4. **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
5. **Hashimoto O, Nakamura T, Shoji H, Shimasaki S, Hayashi Y, Sugino H** 1997 A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. J Biol Chem 272:13835–13842
6. **Hashimoto O, Kawasaki N, Tsuchida K, Shimasaki S, Hayakawa T, Sugino H** 2000 Difference between follistatin isoforms in the inhibition of activin signaling: activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. Cell Signal 12:565–571
7. **Iemura SI, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N** 1998 Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo. Proc Natl Acad Sci USA 95:9337–9342
8. **Bilezikjian LM, Blount AL, Corrigan AZ, Leal A, Chen Y, Vale WW** 2001 Actions of activins, inhibins and follistatins: implications in anterior pituitary function. Clin Exp Pharmacol Physiol 28:244–248
9. **Prendergast KA, Burger LL, Aylor KW, Haisenleder DJ, Dalkin AC, Marshall JC** 2004 Pituitary follistatin gene expression in female rats: evidence that inhibin regulates transcription. Biol Reprod 70:364–370
10. **Moriarty GC** 1976 Immunocytochemistry of the pituitary glycoprotein hormones. J Histochem Cytochem 24:846–863
11. **Tougard C, Picart R, Tixier-Vidal A, Kerdelhue B, Jutisz M** 1974 In situ immunochemical staining of gonadotropic cells in primary cultures of rat anterior pituitary cells with the peroxidase labeled antibody technique. A light and electron microscope study. Histochem J 6:103–104
12. **El Etreby MF, Fath El Bab MR** 1977 Localization of gonadotropic hormones in the dog pituitary gland: a study using immunoenzyme histochemistry and chemical staining. Cell Tissue Res 183:167–175
13. **Dacheux F** 1978 Ultrastructural localization of gonadotrophic hormones in the porcine pituitary using the immunoperoxidase technique. Cell Tissue Res 191:219–232
14. **Herbert DC** 1976 Immunocytochemical evidence that luteinizing hormone (LH) and follicle stimulating hormone (FSH) are present in the same cell type in the rhesus monkey pituitary gland. Endocrinology 98:1554–1557
15. **Pelletier G, Leclerc R, Labrie F** 1976 Identification of gonadotropic cells in the human pituitary by immunoperoxidence technique. Mol Cell Endocrinol 6:123–126
16. **Hymer WC, Evans WH, Kraicer J, Mastro A, Davis, Griswold E** 1973 Enrichment of cell types from the rat adenohypophysis by sedimentation at unit gravity. Endocrinology 92:275–287
17. **Childs GV, Unabia G** 2001 The use of counterflow centrifugation to enrich gonadotropes and somatotropes. J Histochem Cytochem 49:663–664
18. **Childs GV, Unabia G** 2001 Epidermal growth factor and gonadotropin-releasing hormone stimulate proliferation of enriched population of gonadotropes. Endocrinology 142:847–853
19. **Attardi B, Klatt B, Little G** 1995 Repression of glycoprotein hormone α-subunit gene expression and secretion by activin in αT3-1 cells. Mol Endocrinol 9:1737–1749
20. **Alarid ET, Windle JJ, Whyte DB, Mellon PL** 1996 Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development 122:3319–3329

21. **Lavia P, Milco AM, Giordano A, Paggi MG** 2003 Emerging roles of DNA tumor viruses in cell proliferation: new insights into genomic instability. Oncogene 22:650–616
22. **Kim GH, Sung SR, Choi EH, Kim YI, Kim KJ, Dong SH, Kim HJ, Chang YW, Lee JI, Chang R** 2000 Dedifferentiation of conditionally immortalized hepatocytes with long-term in vitro passage. Exp Mol Med 32:29–37
23. **Janeway Jr C, Travers P** 2001 Immunobiology: the immune system in health and disease. 5th ed. New York: Garland; 150–184
24. **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
25. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408
26. **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
27. **Otsuka F, Shimasaki S** 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 143:4938–4941
28. **Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, Vale W, Sawchenko P** 1989 Production and regulation of inhibin subunits in pituitary gonadotropes. Endocrinology 124:552–554
29. **Bilezikjian LM, Leal AM, Blount AL, Corrigan AZ, Turnbull AV, Vale WW** 2003 Rat anterior pituitary folliculostellate cells are targets of interleukin-1β and a major source of intrapituitary follistatin. Endocrinology 144:732–740
30. **Childs GV** 2002 Development of gonadotropes may involve cyclic transdifferentiation of growth hormone cells. Arch Physiol Biochem 110:42–49

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CONCLUDING REMARKS

Our investigations have contributed to a better understanding of the signaling pathway mediating activins induction of FSHβ gene in gonadotropes as well as developing a method for isolating primary gonadotropes to study the role of paracrine and autocrine factors on FSHβ expression. First, these studies have identified that activin stimulates expression of the FSHβ gene through a Smad-indpendent pathway mediated by TAK1. Initial studies in our lab and recently published reports have focused on characterizing Smad3 as the classical protein mediating activin stimulation of the FSHβ gene.

However, our results show that blocking Smad3 signaling pathway did not alter activins stimulation of the FSHβ gene. Furthermore, over expression of Smad3 only affected basal expression and showed no effect on activin induction of the FSHβ gene. Time course studies have also clearly shown that the FSHβ gene shows a delayed response not typical of Smad-dependent genes which are known to be rapidly activated. These data led us to examine TAK1 pathway, which is the protein kinase previously shown to be activated by activins, TGFβ, and BMPs. Therefore, the data shown here indicate that TAK1 MAPKKK is required for activins induction of FSHβ gene. The data also show that downstream target of TAK1, p38-MAPK is required for activins induction of the FSHβ gene. Therefore, the data reveals that activin stimulates FSHβ gene by activating TAK1 initiating a MAPK signaling cascade leading to activation of downstream p38-MAPK. Taken together these studies serve as a platform for future characterization of activins signaling pathway mediated by TAK1 leading to stimulation of the FSHβ gene.

Secondly, we have developed a method for isolating primary gonadotropes, and were able to use the isolated gonadotropes to understand the role of autocrine and paracrine factors in regulation of

the FSHβ gene. The data clearly show that purified primary gonadotropes still express FSHβ suggesting the production of autocrine factors. However, the data shows that paracrine factors play a major role regulating FSHβ expression. The purified gonadotropes provide a model for analyzing the solitary role of factors regulating FSHβ expression in the absence of other cell types.

Remaining Questions:

Fundamental questions for the mediators of the activin signaling pathway in gonadotropes remain unanswered. First, once activin is bound to it's receptor, what are the adaptor proteins linking the activated activins receptor to TAK1 in gonadotropes? It is possible that anchoring proteins such as X-Linked Inhibitor of Apoptosis (XIAP) that was shown to recruit TAK1 to TGFβ receptors in other cell types is also present in gonadotropes and is required for recruiting TAK1 to the activins receptor to initiate the MAPK signaling cascade. It is also possible that the gonadotropes have their own anchoring protein that specifically recruits TAK1 to the activin receptor.

Secondly, Ski and SnoN corepressors have been shown to associate with Smad proteins, and it is still a mystery if they play any role in regulation of FSHβ expression by activin? Smad binding elements have been identified on the FSHβ promoter, and it is possible that bound Smads act as bimodal factors, where they bind Ski or SnoN repressing expression of the FSHβ gene. Upon activin treatment of gonadotropes a signaling cascade would recruit coactivators to the FSHβ promoter that would bind Smads and inducing transcription of the gene.

Lastly, the purified primary gonadotropes still have not been characterized, it is still not known what other genes are being expressed in response to activin? and since activin treatments stimulates FSHβ expression at about 24 hours, it is possible that activin induces the expression of

early genes that establishes a dynamic signaling cascade for stimulation of FSHβ expression. A complete gene expression profiling using microarray technology can help show a general profile of the possible early genes. Another question that needs to be answered: do the gonadotropes express cell surface markers that are unique? It is possible that like many cell types, during development gonadotropes express specific cell surface markers that dictates the developmental stage.