Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone β subunit transcription

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Abstract

Transforming growth factor β superfamily ligands regulate pituitary FSH production and secretion. The best-described examples are the activins and inhibins, which respectively stimulate and hinder *Fshb* subunit transcription in gonadotrope cells. More recently, members of the bone morphogenetic protein (BMP) sub-family were shown to regulate FSH production in a manner analogous to the activins. Here, we used the murine gonadotrope cell line, L β T2, to investigate mechanisms through which BMP2 regulates the *Fshb* gene. Although expressed at low levels in L β T2 cells, *Bmp2* mRNA was readily detected in adult murine pituitary gland. Recombinant BMP2 stimulated *Fshb* promoter-reporter activity, although its effects were weaker than those of equimolar activin A or B. BMP4 stimulated transcription comparably with BMP2, but BMPs 6 and 7 were about tenfold less potent. Remarkably, BMP2 and activin A synergistically upregulated *Fshb* transcription and endogenous *Fshb* mRNA levels in L β T2 cells. Although functionally cooperative, the two ligands appeared to use distinct intracellular mechanisms to mediate their responses because neither ligand altered the timing or magnitude of the other's effects. Receptor overexpression analyses suggested that BMP2 may preferentially signal through complexes of the type II receptor, BMPR2, and the type I receptor, activin receptor like kinase (ALK2; Acvr1), to stimulate *Fshb* transcription. BMP2 rapidly activated the Smad1/5/8 intracellular signaling cascade and Smad8 overexpression potentiated BMP2's effects. In summary, BMPs regulate *Fshb* transcription in L β T2 cells and can amplify the already robust effects of the activins through a distinct signaling mechanism. Because BMP2 is expressed in the adult mouse pituitary, it may act as critical paracrine co-regulator of FSH synthesis by gonadotropes.

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Introduction

The gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are both coordinately and differentially regulated under various physiological conditions. In many cases, singular changes in FSH synthesis and secretion are attributable to actions of transforming growth factor (TGF) β superfamily ligands, the activins and inhibins, in particular. Recent data show that activins stimulate FSH synthesis by upregulating FSH β subunit gene (*Fshb*) transcription, at least in part, through the activation of the receptor-regulated signaling proteins, Smads 2 and 3 (Dupont *et al.* 2003, Suszko *et al.* 2003, Bernard 2004, Gregory *et al.* 2005, Suszko *et al.* 2005, Lamba *et al.* 2006). In the case of the rat and mouse genes, these Smads, in concert with Smad4, bind to a consensus Smad-binding element in the proximal promoter to exert their actions. The promoters in sheep, pig, and human lack this element and therefore mechanisms of activin/Smad action appear to differ in

these species (Bailey *et al.* 2004, West *et al.* 2004, Safwat *et al.* 2005, Lamba *et al.* 2006).

Inhibins suppress FSH through a competitive binding mechanism (Harrison *et al.* 2005). That is, inhibins A and B can bind to activin type II receptors (ACVR2A and ACVR2B) via the β subunits they share with activins. In so doing, they hinder receptor access by activins, leading to decreased *Fshb* mRNA expression. Inhibins bind with significantly lower affinity than the activins, in part because the latter contain two β subunits and can engage two type II receptors simultaneously, whereas the former have only one β subunit and therefore only bind one type II receptor at a time (Cook *et al.* 2005). Inhibins' affinity for type II receptors can be increased significantly in the presence of the co-receptor betaglycan (Lewis *et al.* 2000, Chapman *et al.* 2002). Unlike the activins, inhibins do not recruit the activin type I receptor, ACVR1B (commonly referred to as activin receptor-like kinase 4 or ALK4), into the complex and therefore cannot activate the Smad2/3-pathway (Lebrun & Vale 1997).

Like the inhibins, follistatins also negatively regulate FSH through abrogation of activins' actions. However, rather than competing for receptor binding, follistatins bind directly and irreversibly to activins, burying a large percentage of the ligands' surfaces, including residues involved in receptor binding (Thompson *et al.* 2005). Follistatin-288 (Fst-288) also appears to increase internalization of activins and their degradation through a lysosome-mediated pathway (Sugino *et al.* 1997). Across rodent estrous cycles, circulating inhibins and intra-pituitary follistatins are regulated in a manner consistent with their roles in activin antagonism (Woodruff *et al.* 1996, Besecke *et al.* 1997). In fact, their coordinate decline at or just after the primary gonadotropin surges on the evening of proestrus is argued to provide a permissive hormonal environment for locally produced activins to stimulate *Fshb* subunit transcription, hence generating the secondary FSH surge in rodents.

Although elegant in its simplicity, this model of activin-induced FSH expression was recently challenged by three separate, but related observations. First, additional ligands in the TGF β superfamily, in particular bone morphogenetic proteins (BMPs) 6, 7, and 15 were shown to selectively stimulate FSH in a manner analogous to the activins (Huang *et al.* 2001c, Otsuka & Shimasaki 2002). Secondly, some follistatin isoforms can bind BMPs and hinder their biological activities (Sidis *et al.* 2006). Thirdly, inhibins, once thought to uniquely antagonize activin signaling, were shown to inhibit BMP actions via a ternary complex of betaglycan and type II receptors (Wiater & Vale 2003, Wiater *et al.* 2006). Thus, BMPs might act alone or in concert with activins to stimulate FSH synthesis, and might, like activins, be negatively regulated by inhibins and follistatins.

Whether or not BMPs are bona fide physiological regulators of FSH *in vivo* remains to be determined. If they are, then their intracellular mechanisms of action are likely to differ from those of the activins. Whereas activins signal through Smads 2 and 3, BMPs use the related, but structurally distinct, receptor-regulated Smads 1, 5, and 8 (Miyazono 1999, Miyazawa *et al.* 2002). This raises the intriguing notion that TGF β superfamily ligands might use multiple signaling pathways to coordinately regulate FSH synthesis. Here, we describe a candidate signaling pathway through which BMPs regulate murine *Fshb* transcription in gonadotrope cells.

Materials and methods

Ligands and constructs

Human recombinant (rh-) activin A, activin B, BMP2, BMP4, BMP6, and BMP7 were purchased from R&D

Systems (Minneapolis, MN, USA). Activin A was also generously provided by Dr Teresa Woodruff (Northwestern University, Evanston, IL, USA), as were expression constructs for rat Flag-ActRIIA (Acvr2a), Flag-ActRIIB2 (Acvr2b2), HA-Acvr1 (ALK2), HA-Acvr1b (ALK4), and human Flag-Smad1. Constitutively active HA-ALK2(Q207D) and HA-rat ALK4(T206D) were generated by site-directed mutagenesis. HA-human ALK3(Q233D) was provided by Dr Peter Scheiffele (Columbia University, New York, NY, USA). Human Flag-Smad2 in pCAGGS (Dr E Roberston, Oxford University, Oxford, UK), human Flag-Smad3 and rat Myc-Smad8 (Dr Y Chen, Indiana University, Indianapolis, IN, USA), and human Flag-Smad5 (Dr T Watanabe, Tokyo University, Tokyo, Japan) were generously provided by the indicated investigators. The truncated Smad8 construct was produced by introduction of a premature stop codon just N-terminal to the C-terminal serines by site-directed mutagenesis. The $-1990/+1$ *Fshb*-luc reporter construct and 5' deletions thereof were described previously (Bernard 2004, Lamba *et al.* 2006). The BREX4-luc BMP-responsive reporter (Hata *et al.* 2000) and human BMPR2 expression construct (Liu *et al.* 1995) were provided by Dr Joan Massague (Memorial Sloan Kettering Cancer Center, New York, NY, USA). The $-763/+91$ ovine *Fshb*-luc reporter was generated by subcloning the *SacI/XhoI* fragment from the parental $-4741/+759$ o*Fshb*-luc (gift from Dr W Miller, North Carolina State University, Raleigh, NC, USA) into the same sites in pGL3-Basic. The porcine reporter, $-767/+8$ p*Fshb*-luc, was generated in two steps. First, $-1369/+8$ was PCR amplified from genomic DNA (Novagen, porcine whole blood genomic DNA) using the following primer set and ligated into the *MluI/XhoI* sites of pGL3-Basic: Forward, GCGACGCGTAGCTCAACCTATCCA-TACCGAATC; Reverse: CCCGCTCGAGAAGCTGTGGG-CTGAATCTAGTCTC (restriction sites are underlined). Secondly, the resulting construct was digested with *SacI* to remove sequence upstream of -767 and was re-circularized. All constructs were validated by sequencing.

Cell culture, transfections, and luciferase assays

Immortalized murine gonadotrope L β T2 cells were provided by Dr Pamela Mellon (University of California, San Diego, CA, USA) and were cultured as described previously (Bernard 2004). Cells were plated and transfected in 6- or 24-well plates with Lipofectamine/Plus or Lipofectamine 2000 (Invitrogen) as previously described (Bernard 2004, Gore *et al.* 2005, Lamba *et al.* 2006). Specific treatment conditions are described in the text or figure legends where appropriate. For reporter assays, cells were washed with 1 $\times$ PBS and lysed in 1 $\times$ Passive Lysis Buffer (Promega). Luciferase assays were performed on a Luminoskan Ascent luminometer (Thermo Labsystems, Franklin,

MA, USA) using standard reagents. All treatments were performed in duplicate or triplicate (as indicated), and each experiment was repeated two or more times.

RT-PCR

BMP family member mRNA expression was examined in adult male C57BL6/J mouse pituitary and LβT2 total RNA using a MultiGene-12 RT-PCR Profiling Kit following the manufacturer's instructions using 35 cycles (Super-Array, PM-040B, Frederick, MD, USA). Forty percent of each reaction was resolved on a 1% agarose gel.

BMP receptor, Smad, activin subunit (all 35 cycles), and *Rpl19* (25 cycles) mRNAs were amplified from LβT2 cell, adult male C57BL6/J mouse pituitary, and adult female 129SvEv ovarian total RNA using the primer sets shown in Table 1 and previously described methods (Bernard 2004).

Quantitative PCR

Total RNA was isolated from LβT2 cells in 12-well plates treated with activin A, BMP2 or the two in combination for approximately 24 h. All treatments were performed in triplicate and the experiment repeated thrice. cDNA synthesis and real-time PCR were performed using an ABI7900HT (Applied Biosystems) as previously described (Yuen *et al.* 2002). Ribosomal protein S11 (*Rps11*), α tubulin (*Tuba*), and β -actin (*Actb*) mRNA levels were

assayed in all samples. *Fshb* mRNA measurements were normalized using a robust global normalization algorithm. All control crossing threshold (Ct) values were corrected by the median difference in all samples from actin. All samples were then normalized by the difference from the median Ct of the three corrected control gene Ct levels in each sample, with the value converted to a nominal copy number/cell by assuming 2500 actin mRNA molecules/cell and an amplification efficiency of 93%. Sequences of PCR primers were as follows: *Fshb* forward: GAGAGCAATCTGCTGCCATAG, reverse: CCAGGCAATCTTACGGTCTC; *Rps11* forward: CGTGACGAAGATGAAGATGC, reverse: GCACATTGAATCGCACAGTC; *Actb* forward: AGGTGACAGCATTGCTTCTG, reverse: GCTGCCTCAACACCTCAAC; *Tuba* forward: TGCCTTTGTGCACTGGTATG, reverse: CTGGAGCAGTTTGACGACAC. Data from replicates in individual experiments were averaged and these values used in statistical analysis such that $n=3$ per treatment.

Immunoblots

Whole cell protein extracts were prepared from LβT2 cells treated as described in the text and subjected to immunoblot analyses as previously described (Bernard 2004). Briefly, equivalent amounts of protein were separated by SDS-PAGE (NuPage, Invitrogen) and transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Blots were probed with

Table 1 Primers used in RT-PCR analyses

Gene	Primer orientation	Primer sequence	Size (bp)
<i>Inhba</i>	Forward	5' CTTTCCTCATGCTGCAGGCTA 3'	410
	Reverse	5' CTAGGAGCAGCCACACTCCTC 3'	
<i>Inhbb</i>	Forward	5' AGCTGGCCGTGGTGCCBGTTT 3'	442
	Reverse	5' CACTCCTCCACRATCATGTTG 3'	
<i>Bmpr2</i>	Forward	5' AGGGATGACTTCCTCGCTGC 3'	684
	Reverse	5' AACTGGACGCTCATCCAAGG 3'	
<i>Acvr2a</i>	Forward	5' ATGGGAGCTGCTGCAAAGTTG 3'	439
	Reverse	5' GTGGTACCAAGGAATACAGCAG 3'	
<i>Acvr2b</i>	Forward	5' CGCGGAACATGACGGCGCCCT 3'	464
	Reverse	5' GATGAGAGAGAGGCCCTCCAAT 3'	
<i>Acvr1</i> (ALK2)	Forward	5' TTATACAATGGTCGATGGAG 3'	490
	Reverse	5' TTCAGGCGCTCTTGATTGC 3'	
<i>Bmpr1a</i> (ALK3)	Forward	5' ATGACTCAGCTATACACTTACATCAG 3'	627
	Reverse	5' CAGGTCTTTCAATGATTCTCC 3'	
<i>Bmpr1b</i> (ALK6)	Forward	5' ATGCTCTTACGAAGCTCTGG 3'	491
	Reverse	5' TCCAGCCCAATGCTGTACCG 3'	
<i>Smad1</i>	Forward	5' TTACCTGCCTCCTGAAGACC 3'	361
	Reverse	5' TTCCGCATACACCTCTCCTC 3'	
<i>Smad5</i>	Forward	5' TTCCACCAACCCAACAACGC 3'	419
	Reverse	5' TTTGACAACAATCCCAGGCAG 3'	
<i>Smad8</i>	Forward	5' ACTTCCGGCCAGTTTGCTAC 3'	351
	Reverse	5' TGGGGATCTTGACAGCGGTG 3'	
<i>Rpl19</i>	Forward	5' CTGAAGGTCAAAGGGAATGTG 3'	195
	Reverse	5' GGACAGAGTCTTGATGATCTC 3'	

the indicated antibodies using standard techniques. The pSmad2, pSmad1/5/8, and pp38 rabbit polyclonal antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The β -actin and Inhbb mouse monoclonal antibodies were from Sigma and Diagnostic Systems Laboratories (Webster, TX, USA) respectively. Horse radish peroxidase (HRP) conjugated secondary antibodies were from Bio-Rad and enhanced chemiluminescence (ECL) Plus reagents was from GE Healthcare.

Data analysis

The data from replicate experiments were highly similar and were pooled for statistical analyses. Data are presented as fold-change from the control condition in each experiment. Differences between means were compared using one- or two-way analyses of variance followed by *post hoc* Tukey tests where appropriate (Systat 10.2, Richmond, CA, USA). In some cases, data were log-transformed prior to analyses to control for unequal variances. Significance was assessed relative to $P < 0.05$.

Results

BMP6 and 7 modestly regulate *Fshb* transcription in L β T2 cells

A previous report showed that both BMP6 and 7 stimulated ovine *Fshb* (o*Fshb*) reporter activity in pituitaries of transgenic mice and in transiently transfected L β T2 cells (Huang *et al.* 2001c). Here, we examined regulation of murine *Fshb* (m*Fshb*) promoter-reporter activity by recombinant BMP6 and 7 in L β T2 cells. Activin A dose-dependently stimulated $-1990/+1$ m*Fshb*-luc, but the BMPs only modestly increased reporter activity 1.6- to 1.9-fold at the highest concentration tested, 5 nM (Fig. 1). Similarly, BMP7 failed to stimulate endogenous *Fshb* mRNA levels in L β T2 cells at concentrations up to 100 ng/ml (~ 3.3 nM; data not shown), whereas both activin A and B had potent effects at this and lower doses (Bernard 2004). We confirmed that BMP ligands used here were biologically active (data not shown).

Various BMP family members are expressed in adult pituitary gland and L β T2 cells

We next asked whether other BMP family members might have more potent effects on *Fshb* transcription than BMPs 6 or 7. We used RT-PCR to determine which BMPs are expressed in L β T2 cells and adult murine pituitary, and therefore might regulate FSH endogenously. The patterns of mRNA expression as assessed by RT-PCR differed between the two RNA populations

Figure 1 Activin A, but not BMP6 or 7, strongly regulates *Fshb* transcription. L β T2 cells seeded in 6-well plates were transfected with the $-1990/+1$ m*Fshb*-luc reporter and treated with 0, 0.5, 1, 5 nM recombinant activin A, BMP6, or BMP7 in serum-free medium for approximately 24 h. Cell lysates were collected and assayed for luciferase activity. Data are presented as fold difference from the control condition (0) set to 1 and represent the mean ($\pm$ S.E.M.) of two independent experiments performed in triplicate ($n=6$). Points with letters differ from control cells, and those with different letters differ from one another. Significant stimulation by BMP6 or 7 is indicated by * or † respectively.

(Fig. 2A). Whereas both expressed BMPs 6, 7, and 8b at comparable levels, BMP8a levels were higher in L β T2 cells and BMPs 2, 3, 3b, 4, and 5 were all greater in pituitary gland. Of particular note was the expression of BMP2 and 4 in pituitary, as these ligands have been implicated in pituitary organogenesis and cellular differentiation (Rosenfeld *et al.* 2000). Although they are expressed at very low levels in L β T2 cells, their expression in other pituitary cell types in the adult suggested that these ligands could act as paracrine regulators of gonadotrope cells and we therefore probed their roles in *Fshb* transcription.

BMPs 2 and 4 stimulate murine *Fshb* transcription

L β T2 cells were transfected with $-1990/+1$ m*Fshb*-luc and treated with 100 ng/ml recombinant BMP2, BMP4, BMP6, BMP7, activin A, and activin B for 24 h. Both activins strongly stimulated transcription (Fig. 2B). Both BMP2 and 4 stimulated the reporter significantly, whereas BMP6 and 7 had only mild effects at the same dose, as demonstrated previously (Fig. 1). BMP2 and 4 did not stimulate transcription from the empty reporter vector, pGL3-Basic (data not shown). These data showed that BMP ligands can stimulate *Fshb* transcription in concentration ranges comparable with those of the activins; however, BMP2 and 4 were both less potent than both activin A and B. Because of the similarities between BMP2 and BMP4 we observed here and in additional analyses, we focused on BMP2 in the following experiments.

Figure 2 BMP family members are expressed in pituitary gland and regulate *Fshb* transcription. (A) The indicated BMP family members were amplified from LβT2 cell and adult murine pituitary total RNA by RT-PCR. (B) LβT2 cells seeded in 24-well plates were transfected with the $-1990/+1$ mFshb-luc reporter and treated in triplicate with 100 ng/ml indicated ligands for 24 h. The data are from two independent experiments ($n=6$). (C) Cells were transfected as in B and treated in triplicate with 0.01 or 0.1 μg/ml BMP2, or 0.1 or 1 μg/ml BMP6 or 7 for 24 h. Data are from two experiments ($n=6$). The data for the control group (0 μg/ml) are not pictured so that the remaining points can be presented on a log scale. In B and C, letters indicate significant differences from control. Points with different letters differ from one another.

A previous report indicated that at very high concentrations (1 μg/ml), BMP6 and 7 could stimulate ovine *Fshb* reporter activity (Huang *et al.* 2001c). Here, we examined these high doses and their effects on the

murine promoter relative to BMP2. The data indicated that BMP2 was about tenfold more potent than BMP6 or 7 in stimulating *Fshb* transcription (Fig. 2C).

BMP2 and activin A synergistically stimulate *Fshb* transcription

To gain a clearer understanding of the dynamics of BMP2's regulation of *Fshb* transcription, we transfected cells with the 2 kb mFshb reporter ($-1990/+1$) and stimulated with 25 ng/ml activin A or 100 ng/ml BMP2 for 4, 8, or 24 h. We also included a 24-h treatment group where the two ligands were added together. A greater concentration of BMP2 was used here to facilitate detection of changes occurring at earlier time points. As we reported previously (Lamba *et al.* 2006), activin A time-dependently increased transcription, reaching 14-fold by 24 h (Fig. 3A). BMP2 similarly stimulated transcription in time-dependent fashion, peaking at fourfold at 24 h. At no point was it more potent than activin A. Strikingly, the two ligands had a greater than additive effect (44-fold) on mFshb promoter activity at 24 h. This synergistic activity was both unprecedented and unanticipated, and was therefore explored further.

To examine the specificity of this cooperativity, cells were treated with 25 ng/ml activin A in the presence or absence of 25 ng/ml BMP2, 4, 6, or 7 (Fig. 3B). Both BMP2 and 4 potentiated the activin A response and did so proportionately to their independent effects. BMP6 and 7 alone did not significantly affect reporter activity nor did they augment the activin A response.

We next examined the effects of BMP2 alone and in combination with activin A on endogenous *Fshb* mRNA levels to confirm that the responses we observed were not restricted to reporter gene activity. Activin A stimulated a 12-fold increase in *Fshb* mRNA levels as assessed by quantitative RT-PCR (Fig. 3C). BMP2 alone stimulated a twofold increase, but this was not statistically significant. Importantly, however, the two ligands synergized to increase *Fshb* expression 27-fold, consistent with the reporter gene results.

Activin A and BMP2 synergistically stimulate *Fshb* promoter-reporters of several species

To determine the generality of the results observed here, we compared the actions of BMP2 alone and together with activin A on *Fshb* promoter-reporters from mouse, pig, and sheep. These species were selected because the porcine promoter was shown recently to be highly activin A responsive in LβT2 cells (West *et al.* 2004) and because activin regulation of the ovine *Fshb* promoter has been the subject of intensive study over the years (Huang *et al.* 2001a,b,c, Pernasetti *et al.* 2001,

Figure 3 BMPs and activin A synergistically stimulate *Fshb* transcription. (A) L β T2 cells seeded in 24-well plates were transfected with the $-1990/+1$ mFshb-luc reporter and treated in triplicate with 25 ng/ml activin A or 100 ng/ml BMP2 for 4, 8, or 24 h, or with both ligands for 24 h. Data are from three experiments ($n=9$). Significant differences from control are indicated by letters or asterisk and dagger as described in previous figure legends. (B) L β T2 cells transfected with the $-1990/+1$ mFshb-luc reporter were treated in duplicate with 25 ng/ml activin A plus and minus 25 ng/ml BMP2, BMP4, BMP6, or BMP7 for 24 h. The data are from three independent experiments ($n=6$). (C) L β T2 cells seeded in 12-well plates were treated with 25 ng/ml activin A plus or minus 25 ng/ml BMP2 for 24 h. RNA was collected and endogenous *Fshb* mRNA levels measured by quantitative RT-PCR. All treatments were performed in triplicate and the experiment repeated thrice. Data are presented as mean fold-increase from the control condition (no ligands).

Bailey *et al.* 2004, Safwat *et al.* 2005). 5' Flanking regions of comparable lengths for *Fshb* from the different species were ligated into the pGL3-Basic reporter vector: $-846/+1$ mouse, $-767/+8$ pig, and $-763/+91$ sheep. L β T2 cells were transfected with these reporters or the empty vector and treated with combinations of activin A and BMP2. As shown in Fig. 4, both ligands independently stimulated reporter activity from the promoter constructs of all three species. None of the ligands alone or together affected activity of the pGL3-Basic. The porcine promoter was the most sensitive, and the sheep the least, of the three species tested. In all cases, synergy between activin A and BMP2 was observed, suggesting that this may be a general characteristic of *Fshb* regulation. We observed similar synergism with BMP4 with all three promoters (data not shown). Finally, the murine $-846/+1$ reporter here responded similarly to the $-1990/+1$ reporter in previous analyses, allowing us to use these reporters interchangeably in subsequent experiments.

Activin A and BMP2 do not alter the efficacy or timing of the other ligand's effects

We next sought to establish the mode of cooperation between the two ligand types. First, L β T2 cells were transfected with the 2 kb mFshb reporter and were treated with a constant dose (25 ng/ml) of activin A for 24 h. Some cells were simultaneously treated with varying doses (0.01–100 ng/ml) of BMP2. At doses up to 1 ng/ml, BMP2 had neither an effect on reporter activity nor did it alter the fold activin A response

Figure 4 BMPs and activin A synergistically stimulate the porcine, murine, and ovine *Fshb* promoters in L β T2 cells. L β T2 cells seeded in 24-well plates were transfected with the indicated reporter constructs and treated with 25 ng/ml activin A plus or minus 25 ng/ml BMP2 for 24 h. Treatments were performed in duplicate and the experiment repeated thrice ($n=6$). Each reporter was analyzed using separate two-way (activin $\times$ BMP) ANOVAs. Statistically significant differences are indicated with letters as described in previous legends.

(Fig. 5A). At 10 ng/ml, BMP2 stimulated *Fshb* transcription and appeared to potentiate the activin A effect. However, when normalizing for the increase in 'basal' activity attributable to BMP2, there was no change in the fold activin A response. At 100 ng/ml, BMP2 further increased reporter activity and the overall magnitude of the synergistic effect with activin A, but once again did not alter the fold activin A response. Regardless of BMP2 dose, activin A stimulated reporter activity 10.2- to 11.8-fold. We performed the reciprocal experiment, where a constant 25 ng/ml BMP2 dose was applied and activin A was added from 0.01 to 100 ng/ml for 24 h. Here, activin A dose-dependently stimulated reporter activity (with effects first observed at 10 ng/ml) and synergized with BMP2, but did not significantly alter the fold BMP2 response at any dose (1.7- to 2.3-fold; data not shown).

Next, we examined whether BMP2 altered the timing of activin A's effects on transcription. Cells transfected

with the $-846/+1$ m*Fshb*-luc reporter were treated with 25 ng/ml BMP2 for 24 h with or without 25 ng/ml activin A 24 (concurrently), 8, 6, 4, or 2 h before the end of the 24-h interval. In the presence or absence of BMP2, activin A began to stimulate reporter activity between 2 and 4 h and this timing was not altered by BMP2 treatment (Fig. 5B). As expected, the two ligands synergistically stimulated the *Fshb* promoter, but activin A did not significantly alter the magnitude of the BMP2 response at any time point examined (1.8- to 2.4-fold, Fig. 5B). The reciprocal experiment produced comparable results, with BMP2 stimulating transcription between 2 and 4 h and the timing not being advanced or delayed by activin A (data not shown).

Collectively, these data suggest that the two ligands cooperatively stimulated the *Fshb* gene, but did not appear to do so by increasing cell sensitivity to the other ligand. Rather one ligand reset (increased) the 'baseline' promoter activity and the other produced its characteristic response relative to that baseline. In this way, the two ligands synergized (multiplicatively) in terms of overall stimulation of *Fshb* transcription, but appeared to do so using separable mechanisms (see more below).

BMP2 effects in L β T2 cells are modulated by, but not dependent upon, endogenous activin signaling

Having demonstrated the interaction between exogenous activins and BMPs in *Fshb* regulation, we next asked whether endogenous activin signaling in L β T2 cells played a role in the observed BMP2 effects. Indeed, previous data suggested that endogenous activin signaling might be required for both basal and gonadotropin releasing hormone 1 (GNRH1)-regulated *Fshb* expression in L β T2 cells (Pernasetti *et al.* 2001, Jacobs *et al.* 2003). In the case of BMP2, the question was particularly relevant because the ligand can stimulate transcription of the activin/inhibin β B (*Inhbb*) subunit (Jaatinen *et al.* 2002), and activin B (an *Inhbb* homo-dimer) appears to be the biologically relevant isoform in the pituitary (Corrigan *et al.* 1991). In the case of L β T2 cells, we observed *Inhbb* (Fig. 6A, middle panel), but not *Inhba* (Fig. 6A, top panel), mRNA expression, indicating they have the capacity to make activin B, but not activin A or activin AB. We also observed that 100 ng/ml BMP2 upregulated *Inhbb* protein levels (Fig. 6B) as well as stimulated rat *Inhbb* promoter activity in these cells (data not shown). Therefore, some of BMP2's actions on *Fshb* might not only be attributable to underlying activin B signaling, but also to a direct stimulation of activin's signaling capacity.

In light of these observations, we examined BMP2 activation of *Fshb* transcription in the absence of endogenous activin B signaling. We treated cells with the ALK4/5/7 inhibitor, SB431542 (Inman *et al.* 2002). This compound is a competitive inhibitor of ATP

Figure 5 BMP2 and activin A do not alter the timing or magnitude of one another's responses. (A) L β T2 cells seeded in 24-well plates were transfected with $-1990/+1$ m*Fshb*-luc and treated with 25 ng/ml activin A in the presence or absence of 0.01–100 ng/ml BMP2 for 24 h. The numbers above the bars reflect the fold activin A response at the different BMP2 doses. (B) L β T2 cells seeded in 24-well plates were transfected with $-846/+1$ m*Fshb*-luc and treated with 25 ng/ml BMP2 for 24 h. Subsets of control and BMP2-treated cells were treated with 25 ng/ml activin A for 2, 4, 6, 8, or 24 h. The numbers above the points indicate the fold BMP2 effect at the different times of activin A treatment.

Figure 6 Endogenous activin B contributes to BMP2's stimulation of *Fshb*. (A) *Inhba* and *Inhbb* subunits were amplified from LβT2 cell, adult mouse pituitary (Pit), and ovary total RNA by RT-PCR. Ribosomal protein L19 (*Rpl19*) was used as a loading control. RT+ and RT- refer to samples in which the RT enzyme was included or omitted respectively. (B) LβT2 cells in 6-well plates were treated with the indicated concentrations of BMP2 in serum-free medium for approximately 22 h. Proteins were extracted and *Inhbb* levels detected by western blot under reducing conditions. β-Actin was used as a loading control. Markers at the left are in kDa. (C) LβT2 cells seeded in 24-well plates were transfected with -1990/+1 *mFshb*-luc and treated in triplicate for 24 h with 50 ng/ml activin A, activin B, or BMP2 in the presence or absence of 10 μM SB431542. The data are from two independent experiments ($n=6$). Bars with different letters differ significantly from one another.

binding to the receptors, but does not alter signaling capacity of the BMP type I receptors, ALK2, 3, or 6. In preliminary analyses, we found that at 10 μM, SB431542 completely inhibited activin A- and B-stimulated *Fshb* transcription in LβT2 cells (data not shown and Fig. 6C) and had no effect on BMP2-stimulated BREX4-luc (Hata *et al.* 2000) activity in LβT2 or HepG2 cells (data not shown). When LβT2 cells were transfected with the 2 kb *mFshb* reporter and treated with 10 μM SB431542, we observed a 26% decrease in basal reporter activity and complete inhibition of both activin A and B stimulated transcription. In contrast, the BMP2 effect, while inhibited, was still 2–3-fold greater than basal in the presence of the inhibitor (Fig. 6C, compare bars 2 and 8). These data suggest that

endogenous activin B signaling was not required for the BMP2 effect, although it did contribute to some extent to its overall magnitude.

BMP receptors and receptor-regulated Smads are expressed in LβT2 cells

To better understand the molecular mechanisms through which BMP2 might transduce its effects on *Fshb* transcription, we examined mRNA expression for BMP type II receptors (*Bmpr2*, *Acr2a*, and *Acr2b*), type I receptors (*ALK2*, *ALK3*, and *ALK6*) and receptor-regulated Smads (*Smad1*, 5, and 8) in LβT2 cells and adult pituitary. Consistent with previous reports (Pernasetti *et al.* 2001, Otsuka & Shimasaki 2002, Dupont *et al.* 2003,

Suszko & Woodruff 2006), we readily detected the three type II receptor mRNAs as well as *ALK2* and *ALK3* mRNAs (Fig. 7). One report indicated no expression of *ALK6* in L β T2 cells or mouse pituitary (Otsuka & Shimasaki 2002), whereas more recent work (Suszko & Woodruff 2006) showed that it is expressed at low levels and our data are consistent with the latter report (Fig. 7). We show here for the first time that *Smad1*, *5*, and *8* mRNAs are expressed in L β T2 cells (Fig. 7).

BMP2 can signal preferentially through BMPR2 and ALK2 to regulate *Fshb* expression

To dissect mechanisms of BMP2 action without the confounding influence of endogenous activin B signaling (Fig. 6C), we performed the next series of experiments in the presence of SB431542. Like other TGF β superfamily members, BMPs signal through hetero-tetrameric type II/type I receptor complexes (Allendorph *et al.* 2006). To determine which of the available type II receptors might be used by BMP2, cells

were transfected with the $-846/+1$ *mFshb* reporter and expression vectors for human BMPR2, rat *Acvr2a*, or rat *Acvr2b*, and treated with 25 ng/ml BMP2 for 24 h. In the presence of SB431542, 25 ng/ml BMP2 stimulated a 1.5-fold increase in reporter activity and this fold activation was not altered by *Acvr2a* or *Acvr2b* overexpression (Fig. 8A). Both of these receptors enhance activin A signaling when overexpressed in these cells (data not shown). In contrast, overexpression of BMPR2 significantly augmented the BMP2 response to 2.5-fold (Fig. 8A).

We took a similar approach to assess the potential role of specific type I receptors in the BMP response. In light of the low level of *ALK6* expression in these cells (Fig. 7; Otsuka & Shimasaki 2002), we focused our attention on *ALK2* and *ALK3*. We overexpressed rat *ALK2* or human *ALK3* alone and in combination with *Acvr2a* or BMPR2 with the *Fshb* reporter. Neither type I receptor alone augmented the BMP2 response, nor did *ALK3* in combination with either of the type II receptors (Fig. 8B). In striking contrast, *ALK2* and BMPR2 together upregulated reporter activity in both the presence and the absence of ligand. These effects were specific as *Acvr2a* failed to synergize with *ALK2*. Collectively, these data suggest that BMP2 might preferentially act through complexes of BMPR2 and *ALK2* receptors to regulate the *Fshb* promoter in these cells.

Because BMP2 also binds complexes of BMPR2 and *ALK3* (ten Dijke *et al.* 1994, Liu *et al.* 1995), the functional differences between *ALK2* and *ALK3* in our system may reflect differences in intracellular signaling events mediated by these receptors (e.g. differences in modes of Smad activation, Macias-Silva *et al.* 1998, Chen & Massague 1999). To examine this idea in the absence of confounding effects of differences in relative affinity of BMP2 for BMPR2/*ALK2* versus BMPR2/*ALK3* complexes, we overexpressed constitutively active forms of the two receptors, *ALK2QD* and *ALK3QD*, which can signal in the absence of type II receptors and ligand (Hoodless *et al.* 1996). Remarkably, neither receptor alone stimulated $-846/+1$ *mFshb*-luc activity in L β T2 cells in the presence of SB431542 (Fig. 8C). However, when co-expressed with BMPR2, which had no effect on its own, both receptors stimulated the *Fshb* gene. Importantly, *ALK2QD* was more potent than *ALK3QD* in this assay, consistent with the hypothesis that the two receptors are not equivalent in activating intracellular signaling in this context.

Figure 7 Expression of BMP signaling components in L β T2 cells and adult mouse pituitary (Pit). BMP type I (*ALK2*, *3*, and *6*) and type II receptors (*Acvr2a*, *Acvr2b*, and *Bmpr2*), as well as signaling molecules (*Smads 1*, *5*, and *8*) were amplified from L β T2 cells and adult mouse pituitary total RNA by RT-PCR. *Rpl19* was used as a loading control. RT + and RT – are as described in Fig. 6.

BMP2 preferentially signals through phospho-Smad8/4 to regulate *Fshb* transcription

BMPs most frequently signal via activation (i.e. phosphorylation) of the receptor-regulated Smad proteins; however, Smad-independent signaling pathways have also been described (Derynck & Zhang

Figure 8 BMPR2 and ALK2 overexpression potentiate BMP2-regulated *Fshb* expression. (A) L β T2 cells seeded in 24-well plates were transfected with -846/+1 m*Fshb*-luc and the indicated receptor expression vectors. Cells were then treated in duplicate with 25 ng/ml BMP2 in the presence of 10 μ M SB431542. The data are from three independent experiments and are presented as the fold-stimulation by BMP2 in the presence of each of the receptors ($n=6$). (B) Cells were transfected and treated as described in A, except here different type I (ALK2 or ALK3) and type II receptors (BMPR2 or Acvr2a) were introduced alone and together as indicated. Data are from three independent experiments with treatments performed in duplicate ($n=6$) and are presented relative to the control group, in which no receptors or ligands were included. (C) L β T2 cells seeded in 24-well plates were transfected in triplicate with -846/+1 m*Fshb*-luc and the indicated receptor expression vectors. Cells were then treated with 10 μ M SB431542 for 24 h prior to analysis. The data are from three independent experiments ($n=9$). In all panels, statistically significant differences from the control condition are indicated with asterisk and dagger.

2003). The data above suggested that BMP2 might use ALK2 as its signal propagating receptor and this receptor can activate Smads 1, 5, and 8 (Chen *et al.* 1997, Chen & Massague 1999). We treated L β T2 cells with 50 ng/ml BMP2, in the presence of SB431542, for various periods of time and measured Smad1/5/8 phosphorylation by western blot. As shown in Fig. 9A (top panel), BMP2 stimulated increases in the abundance of two bands detected by the pSmad1/5/8 antibody within 15–30 min, with the smaller of the two bands emerging first. We do not know the exact identities of these proteins as the antibody cross-reacts with phospho-Smads 1, 3, 5, and 8 and Smads 1 and 5

are the same size (465 amino acids). However, based on their relative molecular weights and the fact that BMP2 does not stimulate Smad3 phosphorylation in these cells (data not shown), we predict that the top band is a combination of pSmad1 and pSmad5, and the bottom is pSmad8. Smads 1, 5, and 8 remained phosphorylated through 24-h treatment (data not shown). Although, it is possible that BMP2 activated Smad1/5/8-independent pathways in these cells, it failed to stimulate p38 mitogen activated protein kinase (MAPK) (Fig. 9A, second panel; Kimura *et al.* 2000) or Smad2 phosphorylation (Fig. 9A, third panel). Consistent with its effects on *Fshb* expression, BMP6 was about tenfold less

Figure 9 BMP2 activates the Smad1/5/8 pathway in L β T2 cells. (A) L β T2 cells seeded in 6-well plates were treated with 10 μ M SB431542 and then incubated with 50 ng/ml BMP2 for the indicated times. Protein lysates were subjected to western blot analyses and sequentially probed (top to bottom) with phospho-Smad1/5/8, phospho-p38, phospho-Smad2, and β -actin antibodies. (B) L β T2 cells seeded in 6-well plates were incubated with the indicated concentrations of BMP2 or BMP6 for approximately 1 h. Western blots were performed on whole cell lysates using phospho-Smad1/5/8 (top) and β -actin (bottom) antibodies.

potent than BMP2 in stimulating Smad1/5/8 phosphorylation (Fig. 9B).

We next overexpressed Smads 1, 2, 3, 5, and 8 in L β T2 cells along with the $-846/+1$ m*Fshb* reporter treated with 25 ng/ml BMP2 in the presence of SB431542 for 24 h. In the absence of ligand, Smads 1, 2, 5, and 8 alone had no effects on transcription as we reported previously for endogenous *Fshb* mRNA levels (Bernard 2004; Fig. 10A). Smad3, however, strongly (69-fold) stimulated the promoter (Suszko *et al.* 2003, Bernard 2004, Gregory *et al.* 2005, Suszko *et al.* 2005, Lamba *et al.* 2006). This effect did not require endogenous activin B signaling as it occurred in the presence of SB431542, although overexpressed Smad3 is known to translocate to the nucleus in the absence of active signaling (Reguly & Wrana 2003). The fold BMP2 response was significantly enhanced by Smad8 (18.3- vs 2.1-fold) but not by the other R-Smads. These data suggested that Smad8 might be the preferred signaling protein in the BMP pathway, which is consistent with its rapid phosphorylation after ligand treatment in these cells (Fig. 9).

The R-Smads most often, though not always, act in concert with the co-Smad, Smad4, to mediate their effects (Lagna *et al.* 1996, Levy & Hill 2005, He *et al.* 2006). We, therefore, examined the effects of overexpressed Smads 5 and 8 in the presence and the absence of Smad4. Again, Smad8, but not Smad5, alone potentiated the BMP2 effect (Fig. 10B). Smad4 alone had no effect on basal or BMP2-regulated transcription, but potentiated the Smad8 effect. Smad4 did not significantly alter the Smad5 response and similar results were observed with Smads 1, 2, or 3 (data not shown).

Smad8, like other receptor-regulated Smads, is principally regulated through C-terminal phosphorylation. Indeed, we observed increases in Smad8 phosphorylation in response to BMP2 (Fig. 9). To determine the necessity of this phosphorylation event, we overexpressed wild-type Smad8 and a mutated form that lacks the C-terminal serine residues that are phosphorylated by the activated type I receptor (Smad8 Δ C). Whereas Smad8 potentiated the BMP2 effect, Smad8 Δ C failed to do so. Collectively, these data suggest that BMP2 stimulates the formation of pSmad8/Smad4 complexes that can regulate *Fshb* transcription.

Finally, to determine the time-course of BMP2's regulation of the *Fshb* gene via Smad8, we transfected L β T2 cells with Smad8 and $-846/+1$ m*Fshb*-luc, and then stimulated with BMP2 in the presence of SB431542 for 2, 3, 4, 6, 8, or 24 h. Increases in transcription were detected within 2–3 h, peaked by 8 h, and remained stable through 24 h (Fig. 10D). Thus, the effects mediated through this pathway were both rapid and delimited.

Discussion

The results presented here uncover several previously unappreciated aspects of TGF β superfamily regulation of FSH synthesis. We show that BMP2: (1) stimulates *Fshb* subunit transcription and *Fshb* mRNA levels in L β T2 cells; (2) is approximately tenfold more potent than BMP6 or 7 in producing these effects; (3) synergizes with activin A to robustly upregulate *Fshb* murine, porcine, and ovine promoter-reporter activities; (4) stimulates the *Fshb* promoter using a mechanism that is complementary to but distinct from that used by the activins; (5) preferentially signals through complexes of BMPR2 and ALK2 to regulate the *Fshb* gene; (6) stimulates Smad8 phosphorylation in L β T2 cells and can use complexes of phospho-Smad8 and Smad4 to rapidly upregulate *Fshb* transcription.

Previous analyses showed that BMP6 and 7 could stimulate FSH synthesis but only at high levels of the ligands (Huang *et al.* 2001c). Here, we see that BMP2 or 4 regulate *Fshb* transcription at concentrations comparable

Figure 10 BMP2 rapidly activates *Fshb* transcription via pSmad8/4 complexes. (A) L β T2 cells seeded in 24-well plates were transfected with $-846/+1$ mFshb-luc and the indicated Smad expression vectors (200 ng/well). Cells were then treated with 25 ng/ml BMP2 in the presence of 10 μ M SB431542. Numbers above the bars show the fold effect of BMP2 in the presence of the indicated Smads. (B) Cells were transfected and treated as described in A, except here Smad5 or Smad8 were introduced alone (100 ng/well) or with Smad4 (100 ng/well) as indicated. (C) Cells were transfected as in the previous panels with wild-type or C-terminally truncated Smad8 (100 ng/well). (D) L β T2 cells in 24-well plates were transfected with $-846/+1$ mFshb-luc and Smad8 (200 ng/well) and treated with 25 ng/ml BMP2 for 0, 2, 3, 4, 6, 8, or 24 h in the presence of 10 μ M SB431542. In all panels, data are from three independent experiments with treatments performed in duplicate ($n=6$) and are presented relative to the control group, in which no Smads and/or ligands were included. Significant differences are shown with letters or asterisks as described above.

with those of the activins. Similar results were reported for BMP15 (Otsuka & Shimasaki 2002); however, it is not clear that this ligand is an endogenous regulator of FSH in the pituitary. Although it is expressed in L β T2 cells and adult mouse pituitary, the levels are very low. Moreover, BMP15 mRNA is not detectable in human or sheep pituitaries (Galloway *et al.* 2000, Takeda *et al.* 2003, Faure *et al.* 2005). In contrast, BMP2 and 4 are readily detected in adult mouse pituitary (Fig. 2A; Paez-Pereda *et al.* 2003). Both are also expressed in L β T2 cells, but at very low levels. If this pattern is reflective of pituitary *in vivo*, then both ligands would be predicted to act as paracrine, rather than autocrine, regulators of gonadotropes. We have not yet determined which cell type(s) in the pituitary make BMP2 and 4.

Although higher doses of BMP6 and 7 are required to stimulate FSH synthesis, BMP7 antibodies readily inhibit both ovine *Fshb* reporter activity in transgenic mice and FSH secretion from mouse, rat, and sheep primary pituitary cultures (Huang *et al.* 2001c). The effects of these antibodies on BMP2 or 4 were not investigated, but it is possible that there is some cross-reactivity that could account for their observed actions on FSH. Alternatively, the pituitary may produce heterodimeric proteins, such as BMP2/7 or BMP4/7 and the antibodies might have antagonized their activities. These isoforms are generally more potent than BMP2 or 4 homodimers (Israel *et al.* 1996), so this intriguing possibility merits further consideration. The basis for differential potencies of BMP family members

in this system is unknown, but is currently under investigation.

Perhaps the most surprising result from these analyses was the robust synergistic actions of BMP2 and activin A on *Fshb* transcription and mRNA levels. Overall, BMP2 was weaker than equimolar activin A in regulating gene expression, but together the two ligand types had greater than additive effects (multiplicative in the case of the murine promoter). Detailed analyses showed that neither ligand altered the magnitude or timing of the other ligand's effects. Rather, each appeared to produce independent effects of the *Fshb* promoter that in the aggregate generated profound increases in transcription. These observations suggest that the two ligands likely employ different, non-overlapping mechanisms to generate their effects. If this is an accurate interpretation, then a specific analysis of mechanisms of BMP actions (see more below) should provide an understanding of both their independent and cooperative effects.

The activin A and BMP2 synergism in this system is surprising principally because these ligands more often have been shown to have antagonistic properties. For example, in developing *Xenopus*, activins induce dorsalization of mesoderm, whereas BMPs are ventralizing and can inhibit activin effects (Dale *et al.* 1992, Jones *et al.* 1992). The two ligand types activate different target genes and can inhibit each other's actions via competition for Smad4, which serves as a co-regulator for both activin (Smads 2 and 3) and BMP (Smads 1, 5, and 8) receptor-regulated Smads (Candia *et al.* 1997). More recently, activin A was shown to maintain pluripotency in human embryonic stem cells and to inhibit the differentiating effects of BMPs (Xiao *et al.* 2006).

Consistent with these opposing actions of the ligands and contrary to the results presented here, BMP4 was observed to suppress both basal and activin A-stimulated FSH secretion and *Fshb* mRNA levels in primary sheep pituitary cultures (Faure *et al.* 2005). The reason for the apparently discrepant results is not obvious. The trivial explanation of species differences does not seem to apply here as we specifically examined an ovine promoter-reporter in this system and saw the same pattern of results as in mouse and pig. Perhaps, differences in the intracellular milieu of L β T2 and ovine gonadotrope cells contribute to the different pattern of results. We, in fact, have seen that actions of TGF β 1 are distinct in L β T2 cells and purified murine gonadotropes, whereas the actions of activin A are the same (Gore *et al.* 2005). Another possibility is that the analyses here involve a homogenous transformed cell population, whereas the previous work in sheep used mixed primary pituitary cell cultures. Therefore, in that study, BMP4 could have stimulated FSH in gonadotropes but these actions might have been overshadowed by effects of ligand on other cells in the culture. For

example, BMPs can stimulate follistatin expression and release (Kearns & Demay 2000, Abe *et al.* 2004). If they also stimulate follistatin production in folliculostellate (or gonadotrope) cells, this could explain BMP4 antagonism of basal and activin A stimulated FSH expression (Faure *et al.* 2005). Ultimately, the results obtained here need to be validated in both mixed pituitary and purified gonadotrope cell cultures to resolve these issues.

Receptor overexpression experiments showed that BMP2's actions were potentiated in the presence of BMPR2 and ALK2, but not Acvr2a, Acvr2b, or ALK3. While this approach does not definitively show that these are the receptors mediating the response in this cell line, it suggests that they may represent the preferred receptor combination. Transfection of dominant-negative forms of BMPR2 did not further clarify its role (data not shown). Therefore, we predict that knocking down expression of endogenous receptors (e.g. by RNAi) will be required to confirm their relative roles in this system. We did not examine ALK6 here because an earlier report indicated that it was not expressed in L β T2 cells or adult mouse pituitary (Otsuka & Shimasaki 2002). However, more recent work shows that this receptor is in fact expressed (Suszko & Woodruff 2006), and we confirm this observation here. Given that ALK6 is more similar to ALK3 than ALK2, one would predict that it will not potentiate the BMP2 response. However, ALK3 and ALK6 do not always function identically (Zou *et al.* 1997, Chen *et al.* 1998); therefore, a role for ALK6 in this system merits a thorough examination.

It is interesting to note that none of the receptors examined here altered transcription in the absence of ligand and that ALK2's effects were only observed in the presence of co-expressed BMPR2 (Liu *et al.* 1995). Similarly, a constitutively active form of ALK2 (Q207D) failed to stimulate *Fshb* transcription when expressed alone, but was effective when co-expressed with BMPR2 (Gilboa *et al.* 2000, Nohe *et al.* 2003). These data suggest that BMP2 may signal through pre-assembled receptor complexes to stimulate the *Fshb* promoter. Consistent with this idea, previous reports show that BMP2 can signal through pre-assembled complexes of BMPR2 and ALK3 to activate the Smad1/5/8 pathway and through ligand-induced signaling complexes to activate a Smad-independent, p38 MAPK, pathway (Nohe *et al.* 2002, Hassel *et al.* 2003). Indeed, here we observe that BMP2 rapidly activates Smad1/5/8, but not p38, phosphorylation. It still remains to be determined, however, whether ALK2 forms pre-assembled complexes with BMPR2 as has been described for ALK3.

Although Smads 1 and 5 are expressed in L β T2 cells and appear to be phosphorylated in response to BMP2, neither Smad protein alone nor with Smad4 potentiated ligand-stimulated expression of *Fshb*. Instead,

BMP2 appears to preferentially use Smad8, in concert with Smad4, to regulate this gene. Although these three Smads are structurally related, Smads 1 and 5 are more similar to each other than to Smad8 and the latter can activate transcription more efficiently than the former in some contexts (Pardali *et al.* 2005). Interestingly, BMP4 was shown to more efficiently activate Smad8 than was BMP7 (Aoki *et al.* 2001; similar to our results for BMP2 and BMP6), which could contribute to the differential effectiveness of the ligand types herein. Nonetheless, depletion of endogenous Smad protein levels will ultimately be required to determine their relative functions in BMP-regulated *Fshb* expression (Bernard 2004, Suszko *et al.* 2005, Lamba *et al.* 2006).

The mechanisms through which Smad8 regulates the *Fshb* gene are not currently known. Thus far, we have established that BMP2 acting through Smad8 rapidly stimulates transcription and that its effect plateaus within 8 h. This pattern of activation is distinct from that of activin A, which stimulates a progressive increase in reporter activity through 24 h and beyond (Lamba *et al.* 2006). These observations are consistent with the hypothesized difference in mechanisms of activin A and BMP2 actions. We still do not know where in the *Fshb* promoter BMPs exert their actions or whether the effects are directly mediated by Smad8/Smad4 binding. However, the observations that Smad8 remains phosphorylated through 24-h treatment but its effects on transcription do not increase beyond 8 h may provide some insight into the underlying process. For example, BMP2, via Smad8, may stimulate *de novo* synthesis of a protein directly targeting the *Fshb* promoter and its synthesis may peak within the first 8-h treatment. Alternatively, BMP2 signaling could relieve the *Fshb* promoter from tonic repression that occurs under basal conditions. Such a de-repression mechanism is particularly appealing in light of the pattern of transcriptional activation observed in the presence of overexpressed Smad8.

In conclusion, the data presented here show that BMP2 independently and synergistically with activin A regulates the *Fshb* subunit transcription in mouse. The mechanisms of BMP2 action are not yet completely understood; however, the ligand appears to signal through complexes of BMPR2 and ALK2 to activate a Smad8/4-dependent signaling cascade. Because follistatins and inhibins can antagonize BMP functions as well as those of the activins, the observations reported here may necessitate a revision or reformulation of current models of FSH regulation by TGF β superfamily ligands.

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