Bone morphogenetic protein 2 (BMP2) regulates follicle-stimulating hormone β subunit (*Fshb)* expression in gonadotrope cells

By

Catherine C. Ho

Department of Pharmacology and Therapeutics

McGill University

Montreal, Canada

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Abstract

Proper follicle-stimulating hormone (FSH) synthesis, secretion, and action are required for normal reproductive function in mammals. A major goal of our lab and of this thesis is to reveal the intracellular mechanisms controlling FSH synthesis. The expression of the FSHβ subunit gene (*Fshb*) is rate-limiting in production of the mature dimeric hormone and is regulated by numerous endocrine hormones and paracrine acting factors, including gonadotropin-releasing hormone, sex steroids, and transforming growth factor β (TGFβ) superfamily ligands such as activins. Recent studies suggest that bone morphogenetic proteins (BMPs), a sub-family of TGFβ family ligands, also regulate *Fshb* transcription. BMP2 and BMP4 were further observed to stimulate *Fshb* transcription synergistically with activins. Here, I used the immortalized murine gonadotrope cell line, LβT2, to investigate mechanisms by which BMP2 regulates *Fshb* gene expression. I determined that BMP2 acts through the BMP receptors BMPR1A and BMPR2 to stimulate *Fshb* transcription. The data suggest that BMP2's effect on *Fshb* expression is more significant when acting synergistically with activins, and appears to depend on BMP-stimulated gene expression. cDNA microarray analyses identified inhibitor of DNA binding (Id) proteins as BMP2 gene targets. I showed that Id2 and Id3 are required for BMP2 to stimulate *Fshb* transcription synergistically with activin A. Additionally, Id2 and Id3 physically interact with SMAD3, a major effector of activin signaling, to cooperatively stimulate *Fshb* transcription. Using gonadotrope cells, I showed that BMP2 signals via BMPRIA, BMPR2 and the intracellular signaling proteins SMADs 1 and 5 to stimulate *Id3* transcription. I identified a novel BMP2 response element (BRE) in the proximal murine *Id3* promoter that mediates SMAD1/5-dependent *Id3* transcription. Furthermore, this BRE acts cooperatively with a previously identified distal BRE to mediate BMP2-stimulated *Id3* expression. Overall, my work defines a mechanism whereby BMP2 regulates *Fshb* expression synergistically with activin A. By understanding multiple pathways mediating *Fshb* expression, we will develop a more complete picture of fundamental mechanisms governing reproductive physiology in mammals. Such knowledge may provide the necessary foundation for novel insights into causes of some forms of infertility and may therefore lead to the development of newer and more effective treatments.

Résumé

L'hormone folliculo-stimulante (FSH) est nécessaire pour la reproduction chez les mammifères. Le but principal de notre laboratoire et de cette thèse est d'étudier les mécanismes intracellulaires modulant la synthèse de FSH. L'expression du gène de la chaîne béta (β) de FSH (*Fshb*) est l'étape limitante de la synthèse de cette hormone. Cette dernière est régulée par de nombreuses hormones endocriniennes et de facteurs paracrins comprenant la gonadolibérine, les stéroïdes sexuels et les ligands de la famille de facteur de croissance transformant de type β (TGFβ) tels que les activines. Des études récentes suggèrent que les gènes du développement (BMPs), une sous-famille des ligands de la famille de TGFβ, sont aussi des régulateurs de *Fshb*. Nous avons observé que BMP2 et BMP4 peuvent stimuler la transcription de *Fshb* synergistiquement avec les activines. Ici, j'ai utilisé les gonadotropes murines immortalisées, LβT2, pour étudier les mécanismes par lesquels BMP2 régule l'expression du gène de *Fshb*. J'ai déterminé que BMP2 agit via les récepteurs de BMP, BMPR1A et BMPR2, pour stimuler la transcription de *Fshb*. Les résultats suggèrent que l'effet de BMP2 sur l'expression de *Fshb* soit potentialisé lorsque BMP2 agit en coopération avec les activines, et semble dépendre de l'expression de gènes stimulés par les BMP. La puce à ADN a identifié que l'expression des gènes pour les protéines inhibitrices de l'ADN-liante (*Ids*) est stimulé par BMP2. J'ai démontré que *Id2* et *Id3* sont requis pour que BMP2 stimule la transcription de *Fshb* de façon synergistique avec l'activine A. De plus, Id2/3 et SMAD3, un effecteur important de la signalisation d'activine, font liaison physique pour stimuler coopérativement la transcription de *Fshb*. En utilisant les cellules gonadotropes, j'ai prouvé que BMP2 communique par les récepteurs BMPRIA, BMPR2 et les protéines intracellulaires SMADs 1 et 5 pour stimuler la transcription d'*Id3*. J'ai identifié un élément de réponse du BMP2 (BRE) original dans la séquence du promoteur de l'*Id3*. Additionnellement, ce BRE agit coopérativement avec un autre BRE précédemment identifié pour stimuler l'expression d'*Id3* par BMP2. Dans l'ensemble, mon travail définit un mécanisme par lequel BMP2 régule l'expression de *Fshb* de façon synergistique avec l'activine A. En approfondissant notre compréhension des signaux de transduction multiples qui contrôlent l'expression de *Fshb*, nous développerons une image plus complète des mécanismes qui régente la physiologie de la reproduction des mammifères. Ces connaissances nous permettront de mieux comprendre les causes de l'infertilité, ce qui peut ultimement mener au développement de nouvelles thérapies plus efficaces.

Publications

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Abstracts

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Contribution of authors

Chapter 2

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I was responsible for the experimental design and procedures and for all the data shown in the manuscript. The first draft was written by me. The final version was generated together with Dr. Bernard.

Chapter 3

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I was responsible for the experimental design and procedures and for all the data shown in the manuscript. The first draft was written by me. The final version was generated together with Dr. Bernard.

Chapter 4

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I was responsible for the experimental design and procedures and for all the data shown in the manuscript. The *Bmpr1a* $^{flox/flox}$ mice were generated in the lab of Dr. Yuji Mishina. Xiang Zhou bred the mice and prepared primary cultures. Dr. Paolete Soto taught me how to infect the cultures with adenovirus. The first draft was written by me. The final version was generated together with Dr. Bernard.

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Abbreviations

ACVR1: activin receptor, type I

ACVR1B: activin receptor, type IB

ACVR1C: activin receptor, type IC

ACVR2: activin receptor, type II

ACVR2B: activin receptor, type IIB

ACVRL1: activin receptor-like, type I

ANOVA: analyses of variance

AP1: activator protein 1

AR: androgen receptor

bHLH: basic helix-loop-helix

BMP: bone morphogenetic protein

BMPR1A: BMP receptor, type IA

BMPR1B: BMP receptor, type IB

BMPR2: BMP receptor, type II

bp: base-pair

BRE: BMP response element

cDNA: complementary deoxyribonucleic acid

Cga: gonadotropin α-subunit

ChIP: chromatin immunoprecipitation

CHO: Chinese hamster ovary

Co-SMAD: co-regulatory SMAD

CREB: cyclic AMP resoponsive element binding protein

DHT: dihydrotestosterone

DMEM: Dulbecco's modified eagle medium

dNTPs: deoxynucleotide triphosphates

ER: estrogen receptor

ERK: extracellular regulated MAP kinase

FBS: fetal bovine seurum

FOXL2: forkhead box L2

FSH: follicle-stimulating hormone

FSHβ: follicle-stimulating hormone, β-subunit

FSHR: follicle-stimulating hormone receptor

FST: follistatin

GDF: growth differentiation factors

GnRH: gonadotropin-releasing hormone

GnRHR: gonadotropin-releasing hormone receptor

GPCR: G protein-coupled receptor

hCG: human chorionic gonadotropin

HeLa: human epithelial cervical cancer cells

HepG2: human liver carcinoma cells

HPG: hypothalamic-pituitary-gonadal

HRE: hormone-responsive element

IB: immunoblot

ID: inhibitor of DNA binding

IP: immunoprecipitation

I-SMAD: inhibitory SMAD

JNK: c-Jun NH2-terminal kinase

kb: kilo basepair

LβT2: immortalized gonadotrop cells

LH: luteinizing hormone

luc: luciferase

MAPK: mitogen-activated protein kinase

MH: Mad homology

mRNA: messenger ribonucleic acid

NFY: nuclear transcription factor Y

ng/nl: nanogram/nanoliter

NIH3T3: mouse fibroblast cells

PBS: phosphate buffered saline

PI3K: phosphatidylinositol 3-kinase

PITX1/2: *Paired*-like homeodomain transcription factor 1 and 2

PKA: protein kinase A

PKC: protein kinase C

PLA2: phospholipase A2

PLB: passive lysis buffer

PR: progesterone receptor

qPCR: quantitative polymerase chain reaction

RNAi: ribonucleic acid interference

Rpl19: 60S ribosomal protein L19

rFSH: recombinant FSH

rhCG: recombinant hCG

R-SMAD: receptor-SMAD

SBE: SMAD binding element

siRNA: short interfering ribonucleic acid

SMAD: homolog of mothers against decapentaplegic

SSXS: Ser-Ser-X-Ser

T: testosterone

TAK1: TGFβ-activated kinase 1

TAP: tandem affinity purification

TGFβ: transforming growth factor β

TGFBR1: TGFβ receptor, type I

TGFBR2: TGFβ receptor, type II

TGFBR3: TGFβ receptor, type III

µg/µl: microgram/microliter

WCE: whole cell extract

wt: wild type

General Introduction

1. The hypothalamic-pituitary-gonadal (HPG) axis

Reproductive physiology is controlled by coordinated signals from the brain, pituitary, and gonads, together forming the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.1). Gonadotropin-releasing hormone (GnRH) is synthesized and secreted in pulses by neuroendocrine cells of the anterior hypothalamus in response to stimuli from the brain [1]. The axons of GnRH-producing neurons terminate at the level of the external zone of the median eminence and release GnRH into the hypophyseal portal system [2]. The hormone then travels to the anterior pituitary where it acts on gonadotrope cells to promote the synthesis and secretion of gonadotropins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [3-6] (see Section 3.3.1). GnRH is released in pulsatile fashion and both the frequency and amplitude of pulses change throughout the cycle. Fast GnRH pulses favor LH release, whereas slow pulses favor FSH release, thus allowing GnRH to differentially regulate the synthesis and secretion of LH and FSH [3-5, 7-8]. Moreover, activins, members of the transforming growth factor β (TGFβ) superfamily of secreted ligands, act as paracrine regulators to specifically stimulate FSH secretion without affecting LH secretion [9-13] (see Section 3.4.2), further allowing for the differential regulation of the two hormones. Gametogenesis, the production of gametes, occurs in the gonads and is hormone-dependent. In males, LH stimulates testosterone production, which is crucial for the production of spermatozoa in the testes (see Section 1.3). In females, oocytes are produced in the ovaries; LH and FSH travel through the bloodstream to reach the ovaries, where they act on two hormone secreting cell types, theca cells and granulosa cells [14-15]. LH stimulates the proliferation and differentiation of theca cells, whereas FSH stimulates the proliferation and differentiation of granulosa cells. Granulosa cells are the site of estrogen production. Estrogen acts locally to stimulate oocyte maturation, and is secreted into the circulation to negatively feedback on the anterior pituitary and hypothalamus inhibiting gonadotropin synthesis and secretion (see Section 3.3.3) [16-18]. Granulosa cells are also responsible for inhibin production. Inhibin, another member of the TGFβ superfamily, is also secreted into the bloodstream and travels to the anterior pituitary to competitively antagonize the actions of activin, leading to declines in FSH secretion (Section 3.4.2.1) [11, 19-27]. Each component of the HPG axis is crucial for normal reproductive function, and dys-regulation at any level may result in depressed gonadal function and infertility.

1.1 Hormonal control of the human menstrual cycle

The maturation of the oocyte, followed by its release from the ovary, is a cyclical process [28-29]. In humans, these cycles are termed menstrual cycles; in rodents, they are called estrous cycles. In women, only one gamete fully matures and ovulates in an average 28 day cycle. The menstrual cycle is driven by cyclical changes in hormone synthesis and secretion by the HPG axis.

In utero, the undifferentiated germ cells, oogonia, undergo multiple mitotic divisions [30-31]. At birth, all oogonia are developed into what are known as primary oocytes. After puberty, primary oocytes develop further in selected waves. Oocytes mature in the ovaries enveloped by a supporting structure of cells, together forming the ovarian follicle. Initially, these follicles exist as a pool of **primordial follicles**; each primordial follicle consists of an oocyte surrounded by a single layer of granulosa cells. During a process called follicular recruitment, a cohort of primordial follicles is selected to develop into **pre-antral follicles** [32]; the oocyte enlarges, the granulosa cells proliferate into multiple layers and start to express FSH receptors. The initial stimulus for follicles to start growing from the primordial stage is poorly understood, but growth up to this point is independent of gonadotropin action. In the follicular phase, pre-antral follicles, now expressing FSH receptors, are selected for further maturation under increasing FSH levels [33-37]. These follicles develop into **early antral follicles**, which are characterized by a layer of theca cells located outside the basement membrane. Theca cells express LH receptors and produce androgens in response to LH stimulation [15]. Conversely, granulosa cells express FSH receptors; as the follicle grows, FSH stimulates granulosa cells to produce inhibin and aromatase [38-39]; the latter is responsible for the aromatization of androgens into estrogens. Estrogen and FSH both act to stimulate follicular growth. FSH also induces the expression of LH receptors on granulosa cells. As the antral follicles develop, they produce increasing levels of estrogen and inhibin, which feedback to the anterior pituitary to inhibit FSH synthesis and secretion [40-41]. When one follicle becomes the **dominant follicle**, it can continue to develop under conditions of low FSH stimulation by acquiring more FSH receptors; follicles with granulosa cells expressing LH receptors can develop even in the absence of FSH [42]. The dominant follicle continues to grow by expanding its antrum, a cavity filled with follicular fluid; the follicle at this stage secretes high levels of estrogen and inhibin which further inhibit FSH synthesis. Without FSH support, the other follicles that had begun to mature undergo an apoptotic process, called atresia [33]. The dominant follicle continues to grow without FSH support into what is known as the **mature** (or graafian) **follicle**, and it is now ready for ovulation.

Relatively low levels of estrogen are required for the negative-feedback on gonadotropins. During the estrogen peak at the late follicular phase, estrogen acts by positive-feedback on the pituitary to enhance gonadotrope sensitivity to GnRH. More importantly, estrogen feeds back at the level of the hypothalamus to increase the amplitude and frequency of GnRH pulses, thus driving the increase in LH release, termed the LH surge [43-46]. This LH surge is accompanied by a smaller FSH surge. The LH surge stimulates ovulation; the oocyte is released and travels down the fallopian tube towards the uterus. In the luteal phase, the remains of the ovulated follicle are transformed into the corpus luteum; theca and granulosa cells become luteal cells that secrete estrogen, progesterone, and inhibin in response to LH stimulation. High levels of estrogen, progesterone, and inhibin from the corpus luteum feed back to the hypothalamus and anterior pituitary to inhibit LH and FSH secretion [40-41, 47-51] (Sections 3.3.3 and 3.3.4). There is an inverse relationship between inhibin and FSH levels during both the follicular and luteal phase, supporting that inhibin has an inhibitory effect on FSH secretion. In the absence of pregnancy, the corpus luteum can only survive a few days in the face of minimal LH stimulation. The corpus luteum undergoes luteolysis, resulting in a decrease in estrogen and progesterone production. Low levels of estrogen, progesterone, and inhibin allows for the increase in FSH secretion, stimulating the development of a new cohort of follicles for the next cycle.

1.2 Hormonal control of the rodent estrous cycle

Unlike the menstrual cycle, the estrous cycle in rodents lasts four to five days and is divided into four phases: proestrus, estrus, metestrus, and diestrus [52-54]. Estradiol and inhibins secreted from the growing follicles keep LH and FSH secretion relatively low during metestrus, diestrus, and the morning of proestrus. On the afternoon of proestrus, high estrogen levels coupled with a signal from the suprachiasmatic nucleus, a region in the brain responsible for circadian rhythms, triggers the primary surge of LH and FSH [55-58]. The first phase of FSH (and LH) release is stimulated by increased pulsatile secretion of GnRH from the hypothalamus [59]. This robust surge of pituitary gonadotropins triggers ovulation on the following morning of estrus. During the estrus phase, the female is sexually receptive. FSH levels are maintained high throughout estrus, termed the secondary FSH surge. Inhibin levels decrease during the late evening of proestrus and remain low during the estrus phase [52]. This fall in ovarian inhibin levels creates an environment allowing activins to more readily support the secondary FSH surge. The secondary surge acts to recruit ovarian follicles for the subsequent cycle [60-61]. As the second wave of follicles starts to grow, estrogen and inhibin levels begin to rise again during late estrus and act by negative feedback to suppress gonadotropin synthesis and release. The LH surge during proestrus is accompanied by a progesterone surge [62-64].

Progesterone acts to maintain the uterine epithelium, mammary glands, and the corpus luteum [65-67]. There is also evidence that the increase in progesterone, together with the drop in serum inhibin, contributes to the secondary FSH surge at the pituitary level (Section 3.3.3) [68-70]. Prolactin, a hormone produced in the anterior pituitary, later induces luteolysis in the corpus luteum [71-73]. Some studies suggest that progesterone is also necessary to promote corpus lutem regression [74]. In the absence of pregnancy, the diestrus phase terminates with degeneration of the corpus luteum. However, contrary to humans, the uterus lining is not shed; instead it is reorganized for the next cycle.

1.3 Hormonal control of spermatogenesis

In males, LH and FSH travel through the bloodstream to reach the testis [28-29]. The testis contains Leydig cells, Sertoli cells, and many convoluted seminiferous tubules where spermatogenesis takes place. LH stimulates the proliferation and differentiation of Leydig cells [75-76], whereas FSH stimulates the proliferation and differentiation of Sertoli cells [77-78]. The Leydig cells are the primary site of testosterone synthesis. These cells are interspersed between the seminiferous tubules and express LH receptors. LH stimulates testosterone synthesis and secretion from these cells, which acts locally in the testis to support spermatogenesis [79-80]. Testosterone is also secreted into the circulation, travelling back to the hypothalamus and anterior pituitary to mainly inhibit LH secretion [81-82]. However, testosterone may also have a role in regulating FSH secretion (Section 3.3.2). Sertoli cells line the basement membrane of the seminiferous tubules and surround the developing spermatocytes. Sertoli cells express both FSH and androgen receptors; in response to FSH and local testosterone, these cells produce seminiferous tubule fluid containing nutrients and paracrine signaling factors that stimulate proliferation and differentiation of germ cells [83-84]. Sertoli cells also produce androgen-binding protein which keeps androgen highly concentrated within the testis [85], and inhibin which travels back to the anterior pituitary to competitively antagonize the actions of activin, thus reducing FSH secretion [86-88] (Section 3.4.2.1).

Gametogenesis is hormone-dependent. LH is required to stimulate androgen secretion from theca cells in females and Leydig cells in males. FSH is necessary for promoting aromatase expression in granulosa cells to convert androgen into estrogen. Androgen is crucial for spermatogenesis, whereas estrogen is essential for oocyte maturation. Estrogen and testosterone both act as endocrine hormones providing negative feedback at the hypothalamus and anterior pituitary to inhibit LH and FSH secretion. In addition, FSH is important for stimulating granulosa cell and Sertoli cell proliferation

and differentiation, which are important cells for supporting gametogenesis. Our interest of study is the hormonal control of reproduction in mammals, particularly the role of follicle-stimulating hormone (FSH).

2. The roles of FSH in reproduction

2.1 FSH ligand

As discussed in Section 1, FSH and LH are important regulators of reproductive function in mammals. Both hormones are dimeric glycoproteins composed of disulfide-linked α- and β-subunits. The α-subunit is shared between the FSH, LH, choriogonadotropin hormone (CG), and thyroid-stimulating hormone (TSH); whereas the unique β-subunits confer biological specificity to each of the four hormones [89-91]. The β-subunits are encoded by distinct genes on different chromosomes. The human FSH β-subunit (*Fshb*) gene encodes a 111 amino acid protein, which is highly conserved across species [92-94]. Monoclonal antibodies and synthetic peptides have been used to map the exact residues required for subunit-subunit and subunit-receptor interactions. Although the specificity of FSH lies in the β-subunit, both subunits contact the receptor [95-99].

2.2 FSH signaling in the ovary and the testis

The FSH receptor (FSHR) belongs to the large superfamily of G protein-coupled receptors (GPCRs). In the ovaries, FSH signals via FSHR located on the surface of granulosa cells. FSHR preferentially couples to the G_s protein, which stimulates adenylyl cyclase activity and increase cAMP production. The secondary messenger cAMP activates protein kinase A (PKA) [100-101], which in turn activates downstream signaling pathways, such as the cyclic AMP responsive element binding protein (CREB) transcription factor pathway [102-103], extracellular regulated MAP kinase (ERK) pathway [102], the phosphatidylinositol 3-kinase (PI3K) pathway [104] and the p38 MAPK pathway [103, 105-106]. Activation of the signaling cascades, and cross-talk between them, modulate the expression of FSH target genes. Stimulation of the FSHR by FSH results in the activation of more than 100 different target genes in granulosa cells [107-109]. For example, FSH stimulates increases in LH receptors [110], cell cycle proteins [111], and steroidogenic enzymes [112]. Collectively, FSH signaling acts to promote granulosa cell growth and differentiation.

FSH-mediated PKA signaling in Sertoli cells activates signaling pathways similar to those in granulosa cells. These include, the phosphorylation and activation of CREB [113], PI3K, ERK, and phospholipase A2 (PLA2) [83]. The activation of and cross-talk between these signaling pathways, together with other hormonal signals, such as testosterone, is coordinated to induce the genes necessary to regulate Sertoli cell proliferation/differentiation, and support spermatogenesis [83].

In females, FSH action is crucial for estrogen production by granulosa cells and ovarian follicle maturation. In males, FSH regulates Sertoli cell proliferation and differentiation, thereby indirectly maintaining spermatogenesis. Perturbations in FSH signaling may have a severe impact on ovarian follicular development in females, spermatogenesis in males, and consequently fertility in both genders. The following section will discuss the consequences for fertility when FSH signaling is disturbed.

2.3 Genetic studies of FSH production and action

2.3.1 Murine models

Knock out models have been helpful in determining the function of FSH. In 1997, Kumar et al. successfully generated the *Fshb* knock-out mouse [114]. Female *Fshb* null mice are hypogonadal, infertile, have elevated LH levels and normal estradiol levels. These mice have small ovaries with only primordial and pre-antral follicles; no preovulatory mature follicles or corpora lutea are observed. This indicates that *Fshb* is not required for the initial phases of follicle recruitment and hormone-independent development, but is essential for follicle development beyond the pre-antral stage. Further characterization of the ovaries from these mice by gene expression analysis reveals that the expression of many granulosa cell markers, including *Cpy19* (aromatase) and *Lhr* (LH receptor) mRNA, are reduced [115]. Ovarian folliculogenesis in these animals resumes upon exogenous gonadotropin administration and they produce oocytes similar to those from control mice, suggesting that ovulatory competence in mice is unaffected in the absence of FSH.

Male *Fshb* null mice have decreased testicular size (50%), reduction in sperm number (75%), and decreased sperm motility (40%) [114]. LH and testosterone levels are normal; as such sexual development and progression into puberty are unaffected. In contrast to the females, male null mutants are fertile. FSH is known for its role in supporting Sertoli cell proliferation; consistent with this, *Fshb* knockout mice display a decrease in total seminiferous tubule volume, which may explain the decrease in testicular size. The decrease in total seminiferous tubule volume implies a reduction in

Sertoli cell numbers. Conversely, Leydig cell numbers and the stages of spermatogenesis appear to be normal. The data suggest that FSH is necessary for maintaining normal testicular volume, and for promoting quantitatively and qualitatively normal spermatogenesis, though it may not be absolutely essential for spermatogenesis and fertility, at least in mice.

Fshb and *Fshr* knockout mice display similar phenotypes. Female *Fshr* knockout mice are sterile, have thin uteri as a result of low estrogen levels, and small ovaries devoid of mature follicles. Males *Fshr* knockouts exhibit oligospermia, small testes, and low Sertoli cell numbers, but initially appear to exhibit normal fertility [116]. Later analyses revealed that *Fshr* knockout males have reduced fertility and delayed puberty [117]. Expectedly, FSH levels are elevated in both sexes due to the lack of negative feedback on FSH expression.

Interestingly, high levels of FSH in male mice can lead to infertility, as seen with the over-expression of FSH in transgenic mice [118]. Males have normal sized testes, increased sperm production, elevated testosterone levels, and no testicular defects, except for the seminal vesicles which are twice their normal size due to high testosterone levels. The reason for their infertility is unknown but may result from functional incompetence of the spermatozoa/semen or altered reproductive behaviour. Over-expression of FSH in female mice results in elevated estradiol, progesterone, and testosterone levels, and the mice develop hemorrhagic and cystic ovaries. These mice die due to extreme bladder and kidney enlargement and obstruction between 6-13 weeks postnatally.

From these experiments, we have learned that FSH and/or FSH signaling is required in female mice for follicle development beyond the pre-antral stage. In male mice, FSH is not absolutely required for fertility, but is required to support Sertoli cell growth and to attain full reproductive potential. Interestingly, over-expression of FSH in transgenic mice results in the eventual death of females and infertility in males, stressing the importance of maintaining an optimal FSH expression level. FSH synthesis must thus be tightly regulated to ensure normal fertility in mice.

2.3.2 Human mutations

Mutations in the human *FSHB* and *FSHR* genes are extremely rare, but have provided invaluable information to our understanding of FSH function in humans. Described below are examples of inactivating mutations in the *FSHB* and *FSHR* genes.

Mutations in *FSHB* have been reported in three female patients with low or undetectable circulating FSH, high LH levels, primary amenorrhea, impaired breast development, follicular maturation arrest,

and infertility (Table 1.1) [119-120]. In one case, the patient was homozygous for a two base pair (GC) deletion at codon 61 (Val61X) [119]. This mutation results in a frame-shift mutation which completely alters the amino acid sequence of the FSH β-subunit and produces a premature stop codon leading to the synthesis of a truncated FSHβ protein. In the second case, the patient was a compound heterozygote; she carried the same Val61X mutation on one allele and a second mutation (Cys51Gly) on the other allele [120]. Loss of this cysteine results in aberrant folding, synthesis, and secretion of the FSHβ protein. In the third case, the patient was homozygous for a one base pair (G) deletion at codon 79 of the *Fshb* gene (Ala79X) [121]. Similar to the first case, this deletion also results in a frame-shift mutation producing a truncated protein. All three mutations are predicted to impair dimeric hormone assembly. These data suggest an essential role for FSH in puberty and fertility in women.

Mutations in the *FSHB* gene have been identified in men with azoospermia (no sperm in the ejaculate) and infertility [122-123]. In a less severe case, the patient had azoospermia, normal puberty, small testes, high LH levels, and normal testosterone levels [122]. Sequencing of his *FSHB* gene revealed a homozygous Cys82Arg missense mutation predicted to interfere with dimer formation. *In vitro* analysis confirmed that this mutation results in an *FSHB* gene that is unable to produce immunoreactive and bioactive FSH dimers [124]. In a more severe case, the patient had azoospermia, did not undergo pubertal development, had very small testes, and high LH [123]. This patient carried the same Val61X mutation described above, resulting in a truncated FSHβ protein. These data suggest that whereas FSH may not be need for fertility in male mice, it is fundamentally required for spermatogenesis in human males. However, it should be noted that in some men with isolated FSH deficiency, but no known *FSHB* mutations, normal fertility or variable degrees of oligospermia have been reported [125-126]. These reports suggest a less crucial role for FSH in human spermatogenesis, though it is possible that low levels of FSH (e.g., below the limit of detection) may be sufficient for spermatogenesis in these individuals.

Patients with mutations in the *FSHR* tend to have a phenotype less severe than patients with mutations in *FSHB*. This is mainly because these mutant receptors retain some residual activity. One-third of women identified with hypogonadotropic ovarian failure (HOF) in northern areas of Finland have a homozygous Ala189Val missense mutation in their FSHR [127]. Characteristics of these patients include primary amenorrhea, variable development of secondary sex characteristics, and high levels of serum gonadotropins. The ovaries of these women have varying follicle sizes; some patients only have primordial follicles, whereas others have pre-antral, antral and even mature follicles. However, no corpora lutea were observed, indicating that ovulation did not occur. *In vitro* assays

show that the mutated FSHR is impaired in its trafficking to the plasma membrane, significantly impairing FSH signaling [128]. In another case, an Armenian woman displayed a relatively less severe phenotype: secondary amenorrhea, normal breast development, variably low estradiol, relatively high testosterone levels, and elevated gonadotropins [129]. Ovarian histology demonstrated normal development up to the pre-antral follicle stage. This patient was a compound heterozygote for Ile160Thr and Arg573Cys mutations in FSHR. Both mutations produce receptors partially deficient in signaling. The Ile160Thr mutation produces in a receptor with impaired ligand binding, whereas Arg573Cys reduces FSHR signaling activity upon ligand binding. Nonetheless, residual signaling is observed in receptors carrying either mutation.

Males carrying the homozygous Ala189Val missense mutation in their FSHR have poor sperm quality, ranging from severe to moderate oligozoospermia [130]. These men undergo normal pubertal growth, have reduced testicular volume, normal testosterone levels, normal LH levels, high FSH levels, and two of the five men are fertile. This mutation, though it completely blocks FSH action *in vitro* and also severely affects female fertility, seems to be less severe in men. This suggests that quantitatively normal FSH signaling may not be an absolute requirement for spermatogenesis, but that it is needed for the quantitative and qualitative maintenance of normal spermatogenesis.

Women with inactivating mutations in the *FSHB* and *FSHR* genes display similar phenotypes as female *Fshb* and *Fshr* knockout mice; both are infertile and unable to produce mature ovulatory follicles. These data suggest that early phases of follicular maturation are independent of FSH signaling, but that FSH is crucial for the final stages of maturation. FSH-deficient women can be treated with FSH resulting in successful pregnancy [131-133]; however, there is currently no method (e.g., chaperones or allosteric modulators/agonists) to activate the mutated FSHR. *FSHB* or *FSHR* mutations in men that result in defective FSH signaling display a phenotype that is dissimilar to that found in male *Fshb* and *Fshr* knockout mice. Although sperm production is impaired in both species, some human cases displayed a complete loss in fertility but this was never the case in mice. Moreover, in other cases, men that lack FSH action seem to have impaired, but not complete absence of fertility. With such discrepancies between different studies, it is unclear whether FSH is essential for male fertility. FSH alone can maintain spermatogenesis in monkeys rendered pharmacologically hypogonadotropic by a GnRH antagonist [134]. Also, rodents seem to be able to maintain spermatogenesis with testosterone (T) alone [131]. It is possible that, depending on the species, the absence of one hormone (FSH or T) can be compensated by the function of the other in concert with other hormones and paracrine regulators, to maintain spermatogenesis. Overall, we can conclude that

although FSH is not required for spermatogenesis and fertility, it is likely requisite for the quantitative (and perhaps qualitative) maintenance of normal spermatogenesis.

2.3.3 Targeting FSH signaling therapeutically

As mentioned in the previous section, FSH can be used to treat women with FSH deficiency. It was documented as early as 1958 that treatment with FSH partially purified from human pituitary followed by human chorionic gonadotropin (hCG), a protein structurally and functionally similar to LH but with a longer half-life, induces ovulation in infertile and amenorrheic women [135]. Later, another group showed that treatment with gonadotropins isolated from the urine of post-menopausal women, followed by hCG, also induced ovulation [136]. Follicular development only requires tonic low levels of LH; however, gonadotropin preparations from post-menopausal women contain high levels of LH, which may increase the risk of premature luteinization and follicle atresia. Therefore, women treated with purified FSH preparations have a higher rate of pregnancy than those treated with mixed gonadotrophin preparations [137-138]. FSH acts to promote follicle development, whereas hCG induces ovulation. Exogenous FSH treatment, which does not mimic the normal pattern of endogenous FSH release (Section 1.1), commonly results in the ovulation of multiple follicles and, therefore, multiple gestations [137]. Today, recombinant FSH and recombinant hCG (rFSH and rhCG) provide purer and safer product in assisted reproduction [132-133, 139]. rFSH and rhCG are also used in *in vitro* fertilization [140-142].

There are conflicting data concerning a compulsory role for FSH in male fertility (Section 2.3.1 and 2.3.2). Nonetheless, one group performed several studies assessing the possibility of treating infertile oligospermic men with rFSH [143-146]. Results from their studies remain controversial. The majority of infertile oligospermic patients receiving the treatment did not show modifications in sperm parameters. However, some men displayed an increased sperm count and were able to induce more pregnancies than other men in the study receiving the same treatment, suggesting that rFSH is only effective in select patients. At present, the data do not support treating men with idiopathic oligospermia with rFSH.

On the other hand, FSH receptor antagonists and antibodies targeting the FSH receptor or FSH are being developed as novel methods of contraception. Synthetic peptides corresponding to a partial amino acid sequence of the human FSH β-subunit or the human FSH receptor inhibit FSH binding and FSH-stimulated steroidogenesis in rat Sertoli cells [147-149]. More recently, small molecule FSHR antagonists have also been developed; such antagonists are advantageous because they are not

as easily degraded as peptide antagonists. These nonpeptide antagonists inhibit FSH-stimulated steroidogenesis *in vitro,* as well as follicle growth and ovulation in rats and *ex vivo* murine models [150-151]. Antibodies developed in female ewes or mice to target the FSHR inhibit FSH-induced cAMP production in Chinese hamster ovary (CHO) cells expressing the porcine FSHR [152]. These antibodies can also reduce the ovulation rate in ewes and impair fertility in female mice. Finally, the most convincing data supporting contraceptive vaccines for males are from studies in non-human primates. Monkeys immunized with ovine FSH produce low numbers of poor quality spermatozoa. Whereas these animals maintain normal testosterone levels, they are incapable of impregnating normal cycling females [153-154]. Simultaneously, this study supports that FSH is essential for male fertility, at least in primates. Based on the accumulated data, methods targeting FSH signaling may present feasible contraceptives for men in the future. Testosterone production is not dependent on FSH, whereas estrogen production in females is greatly dependent on the action of FSH; therefore, targeting FSH in females would lead to estrogen suppression and associated adverse effects in bone and the cardiovascular system [155-164]. Consequently, such a contraceptive strategy would not represent an improvement over existing forms of hormonal contraception in women.

The present section described the role of FSH in follicle development and spermatogenesis under physiological conditions. It has also elucidated the pathophysiology of deficient or dysregulated FSH signaling in mice and in humans, demonstrating that FSH is crucial for normal fertility in both males and females. In addition, the current literature involving therapeutics that target FSH signaling was reviewed. FSH expression fluctuates during the menstrual cycle allowing selection, development, and atresia of follicles during different stages of the cycle. Although FSH in males fluctuate less, numerous hormones and paracrine factors tightly regulate FSH expression in both genders. The following section will focus on what is known to date about the regulation of FSH synthesis.

3. Regulation of FSH synthesis

Production of the FSH β-subunit is rate-limiting in the synthesis of the mature FSH hormone. Upon synthesis, FSH is sorted through constitutive secretory pathway. Thus, the release of FSH is directly coupled to its synthesis, and correspondingly the main target for regulation is *Fshb* expression [165-167]. *Fshb* gene transcription is under stringent regulation by many different factors. GnRH plays a critical role in regulating both LH and FSH secretion; however, as described in Section 1 above, the expression pattern of the two is very different. Previous studies have shown that many other factors are specifically involved in controlling FSH synthesis. Such factors include TGFβ family ligands and

sex steroids, which may act together with GnRH to regulate *Fshb* transcription. The regulation of FSH synthesis/secretion requires complex integration of numerous signals. Dissecting and understanding the contribution of each of these signaling pathways will provide insight into the control of reproductive function, which may lead to the development of therapies for infertility. In this section, I will first describe the models that have been used thus far to study the different signaling pathways that regulate *Fshb* transcription, followed by a detailed discussion of each regulator and its effect on *Fshb* expression.

3.1 Models for studying *Fshb* regulation

Until recently, very little was known about *Fshb* transcriptional regulation. This was largely due to the lack of a differentiated gonadotrope cell line that produces *Fshb* endogenously. Therefore, heterologous cells, primary cell cultures, and transgenic mice were previously employed to study transcriptional regulation of the *Fshb* gene. In 1996, an immortalized murine gonandotrope cell line (LβT2) was developed [168]. Later, these cells were recognized to express *Fshb* endogenously in response to GnRH or activin A stimulation [169-170]. Since then, much progress had been made in the field of *Fshb* transcriptional regulation. Additionally, in 2004, a transgenic model was developed to permit rapid, efficient isolation of murine gonadotropes thereby allowing for the study of isolated primary gonadotropes at approximately 95% purity [171]. In the following section, I will describe how each of these models has been used or developed, as well as their relative strengths and weaknesses.

3.1.1 Heterologous cell systems

Before the development of a homologous cell system, heterologous cells were the only resource available for *in vitro* mechanistic studies, and much of the original insight into *Fshb* regulation was derived from analyses in this system. In particular, we learned a considerable amount about *Fshb* regulation by GnRH (Section 3.2). To study *Fshb* transcriptional regulation by GnRH, an ovine *Fshb* promoter-reporter was transfected into a heterologous cell system, for example, human epithelial cervical cancer (HeLa) cells, engineered to express the GnRH receptor (see Section 3.2) [172]. However, because specific sets of proteins, such as transcription factors, kinases, G proteins, and receptors may be expressed exclusively in gonadotropes, the use of heterologous cells may not accurately reflect gonadotrope-specific responses. For example, *in vitro* data suggest a role for GnRH-induced activator protein 1 (AP1) binding to the ovine *Fshb* promoter; however, *in vivo* data

fail to support this model (see Section 3.2). Today, heterologous systems are still being used to formulate and test predictions of the homologous model. In cases where homologous cell lines are difficult to work with, heterologous systems can be used to generate hypotheses which can be later tested in a homologous system. For example, in tandem affinity purification (TAP) experiments, when stable cell lines are difficult to attain using homologous cells, one can perform the screening step using a heterologous cell line, and later confirm the involvement of specific targets using a homologous cell line (see Chapter 5, Section 5.2).

3.1.2 Primary pituitary cultures

Primary pituitary cultures from many different species including mice, rats, sheep, and fish (goldfish, salmon, eel, tilapia, zebrafish) have been used to study the regulation of gonadotropin expression [173-180]. Much of what we know about *Fshb* regulation by activins and steroids stem from primary pituitary cultures. However, there are several inherent problems in using pituitary primary culture preparations. Given the size of the pituitary, many animals are required for each experiment performed in primary cultures. Only 5-10% of the cells in the anterior pituitary gland are gonadotropes [181]; numerous other cell types populate the anterior pituitary, and their specific paracrine effects on FSH synthesis and secretion cannot easily be determined. Also, having multiple endocrine cell types in the primary pituitary culture preparation makes it difficult to differentiate gonadotrope specific responses over the background activities of the other cells. Therefore one must keep in mind the possible effects of neighbouring cells when interpreting data from primary cultures. Finally, primary cells do not transfect well; therefore, they are not amenable to the kind of mechanistic studies more readily conducted in cell lines. Nonetheless, primary cultures may represent *in vivo* models more accurately than cell lines, making them a valuable resource for studying *Fshb* expression.

3.1.3 Transgenic mice

As described in Section 2.3.1, transgenic mice are exceptional tools for determining the physiological functions of certain genes and their protein products. Specific genes in gonadotropes can be knocked out or knocked in, allowing one to assess their roles in *Fshb* expression and fertility. However, transgenic mice also do not allow detailed analyses of signaling pathways or DNA-protein interactions. Moreover, the construction of transgenic models is a very laborious and time consuming procedure. Nonetheless, models developed in cell lines my not reflect what happens *in vivo*. For

example, data from cell lines suggest a role for ERK1/2 in GnRH-stimulated *Fshb* transcription; however, *in vivo* data do not support this model (see Section 3.2). Therefore, it is common (and recommended) practice to use transgenic mice as an *in vivo* validation of *in vitro* models.

3.1.4 Homologous cell system

Two immortalized gonadotrope cell lines were developed in transgenic mice using an SV40 large T-antigen linked to the 5' promoter regions of the human *CGA* (gonadotropin α-subunit) or rat *Lhb* genes [168]. αT3-1 and LβT2 cells were derived from pituitary tumors in mice bearing the *CGA* and *Lhb* transgenes, respectively. The expression of the α-subunit occurs at embryonic day 11.5 (E11.5) of murine gestation, whereas the expression of LHβ occurs at E16.5, and FSHβ at E17.5 [181]. As a result, the αT3-1 cell line is argued to reflect gonadotropes at an earlier stage in the differentiation program. That is, they express markers of early [*Cga, Gnrhr*, and *Nr5a1* (steroidogenic factor 1, or SF-1], but not terminal differentiation (*Lhb* or *Fshb* subunits). LβT2 cells reflect a more differentiated murine gonadotrope cell line and express *Cga, Gnrhr, Nr5a1*, and *Lhb* under basal conditions. These cells can further secrete LH in response to GnRH stimulation [168]. Moreover, LβT2 cells were later observed to express *Fshb* mRNA in response to activin A (and GnRH to a lesser extent) stimulation [169-170], providing a valuable tool for studying *Fshb* transcriptional regulation. In addition to containing the full complement of factors required for basal and hormone-regulated expression of the gonadotropin subunits, LβT2 cells are advantageous because they can be easily expanded and maintained in culture for several passages. LβT2 cells are also amenable to transfection studies using standard molecular biology techniques, thus enabling over-expression and knockdown of specific factors. As seen in subsequent sections, such manipulations in LβT2 cells has allowed researchers to study the pathways involved in GnRH-, androgen-, estrogen-, progesterone-, and activin-mediated *Fshb*-regulation (Sections 3.2, 3.3 and 3.4). The availability of LβT2 cells has greatly expanded our knowledge of *Fshb* transcriptional regulation and these cells are still intensively used today as they remain the only homologous cell line available for such studies.

3.1.5 Purified primary gonadotropes

To avoid some of the concerns in using whole pituitary cultures (see Section 3.1.2), one can use purified gonadotropes to study the effect of exogenous ligands in *Fshb* transcription. Several labs have reported methods to enrich gonadotropes from mixed cell populations in the pituitary. Childs and Unabia described a counterflow centrifugation method for enriching gonadotropes from rat

pituitaries; however, the procedure is time consuming, and requires many animals [182]. Recently, Wu *et al.* developed a protocol for purifying gonadotropes from transgenic mice [171]. These mice were genetically engineered to contain 4.7kb of the ovine *Fshb* promoter linked to a cDNA encoding the cell surface antigen, H2Kk. H2Kk is a major histocompatibility protein absent in most murine strains used for transgenic work. It lacks protease-sensitive sites on its extracellular amino terminus, so it is not digested by enzymes used to disperse mammalian cells. Furthermore, the H2Kk used here lacks an intracellular carboxyl terminus, so it has no intracellular signaling ability to interfere with normal cell functions. Another advantage of expressing H2Kk on the cell surface of gonadotropes is the availability of a commercial technique that uses magnetic immuno-microbeads to rapidly and efficiently purify gonadotropes from mixed pituitary cultures. By immunostaining for FSH, this group reported that this method can isolate gonadotropes at up to 95% purity. Furthermore, treatment of these purified cells with activin A can increase FSH expression by 480% above basal levels. This method thus allows for the study of *Fshb* regulation in a more physiological context. However, H2Kk gonadotropes cannot be purified in sufficient quantities to perform studies of the kind performed in LβT2 cells and many mice are needed to generate sufficient cells for experimentation.

Although each of the available systems to study *Fshb* transcriptional regulation has its shortcomings, these systems can complement each other to definitively establish a role, or lack thereof, for a target of study. Each of these systems has aided and will continue to aid in the identification of *Fshb*-regulatory factors and the components involved in their signaling pathways. In the following sections, I will describe the main known *Fshb* regulators and their associated signaling mechanisms.

3.2 Regulation of *Fshb* expression by GnRH

GnRH, released in pulses from the hypothalamus, is an important stimulator of *Fshb* synthesis [7-8]. GnRH acts on the GnRH receptor (GnRHR), a GPCR. Signal transduction through GnRHR occurs through the activation of G proteins, G_q/G_{11} [183], which subsequently activate phospholipase Cβ. Phospholipase Cβ cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) into inositol triphosphate (IP3) and diacylglycerol (DAG), the latter being an activator of protein kinase C (PKC) [184-185]. GnRH-activated PKC promotes *Fshb* transcription through the activation of MAPK kinase pathways, such as ERK1/2, JNK (c-Jun NH2-terminal kinase), and p38 [185-189].

GnRH stimulation induces immediate-early response genes resulting in the synthesis of transcription factors, such as activator protein 1 (AP1), to promote *Fshb* transcription. AP1 consists of Fos (c-fos,

FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) dimers [190-191], and its expression is activated by GnRH-stimulated MAP kinases. *In vitro* studies suggest that the ERK1/2 pathway is important for GnRH-mediated activation of *Fshb* transcription in LβT2 cells [188, 192-193]. Conversely, *in vivo* data do not support a role for ERK1/2 in the regulation of *Fshb* transcription. Gonadotrope-specific ERK1/2 knockout mice display no impairments in plasma FSH and pituitary *Fshb* mRNA levels. Furthermore, female knockout mice demonstrate normal follicular development [194]. A possible explanation could be the availability of other GnRH-activated kinases that may compensate for the loss of ERK1/2 to activate AP1 expression. *In vitro* data suggest that JNK and p38 MAPK pathways also contribute to AP1 expression and therefore *Fshb* expression in LβT2 cells [188, 192-193]. *In vivo* experimentations will be required to confirm their importance.

Several potential AP1 binding sites exist in the mammalian *Fshb/FSHB* proximal promoters (Figure 1.2), though there appear to be differences in their relative importance between species. Two AP1 binding sites in the ovine promoter mediate GnRH induction of *Fshb* transcription [172]. Fos and Jun proteins bind to these sites and stimulate *Fshb* promoter-reporter activity [195]. Interestingly, the GnRH-responsive murine *Fshb* promoter is deficient in both the AP1 sites identified in the ovine promoter. Instead, an AP1 half site exists in the murine promoter, located juxtaposed to an NFY (nuclear transcription factor Y) site; both of these elements are required for the induction of the murine *Fshb* reporter by c-Fos or c-Jun over-expression, as well as for full GnRH responsiveness in LβT2 cells [196]. Additionally, AP1 physically interacts with NFY, further supporting that AP1 and NFY act together to regulate *Fshb* transcription. The human *FSHB* promoter shares the distal AP1 site identified in the ovine *Fshb* promoter, and the AP1/NFY site identified in the murine promoter [193]. Both of these sites contribute to GnRH induction of the human *FSHB* gene. Furthermore, expression of a dominant negative Fos inhibits GnRH-induced murine and human *Fshb*/*FSHB* promoter activities, further suggesting that GnRH induction of *Fshb*/*FSHB* transcription is mediated by AP1 proteins [185, 196]. Collectively, *in vitro* data suggest a role for AP1 and AP1 sites in the induction of *Fshb*/*FSHB* expression by GnRH, though the *cis*-elements involved may be species-specific.

In vivo studies do not support a role for AP1 in the regulation of *Fshb/FSHB* expression [197]. Transgenic mice were engineered to express the luciferase (luc) reporter under the control of the ovine *Fshb* promoter. This transgene, specifically expressed in pituitary gonadotropes, is regulated similarly to the endogenous murine *Fshb* gene. Surprisingly, transgenic mice expressing ovine *Fshb*-luc with both AP1 sites mutated respond to GnRH agonist and GnRH immunoneutralization similarly

to that of wild-type mice. This suggests that the AP1 sites may not be required for *in vivo* regulation of *Fshb* transcription by GnRH. The *in vivo* relevance of the AP1/NFY site in the murine *Fshb* promoter has not yet been assessed.

Despite the high sequence conservation between the murine and rat *Fshb* promoter, the *trans*-acting factors involved in the regulation of *Fshb* transcription by GnRH appear to be different. A study with the rat promoter suggests that AP1 expression and binding do not affect GnRH-induced *Fshb* promoter activity [198]. Whereas AP1 and NFY bind to the murine *Fshb* promoter, binding of these proteins cannot be detected in the corresponding region of the rat *Fshb* promoter. Instead this region of the rat promoter appears to bind the CREB and upstream transcription factor (USF)1/2 both *in vitro* and in LβT2 cells. Moreover, siRNA-mediated knockdown or expression of a dominant negative CREB construct significantly reduces the GnRH effect. These observations further support that the mechanism by which GnRH modulates *Fshb* expression may be species-specific.

Much effort has been dedicated to understanding mechanisms underlying GnRH-mediated *Fshb* transcription. Current *in vitro* data support the notion of a species-dependent mechanism in the regulation of *Fshb*/*FSHB* transcription by AP1. However, *in vivo* studies do not support the model that GnRH-mediated *Fshb* expression is dependent on the AP1 sites in the ovine *Fshb* promoter. Whether AP1 or AP1-sites are necessary for GnRH-induced *Fshb* transcription in other species remains to be elucidated *in vivo*. Regulation of *Fshb* transcription by GnRH is complex and, at present, incompletely understood.

3.3 Regulation of *Fshb* expression by steroids

As discussed in Section 1.1, FSH promotes androgen, estrogen, and progesterone synthesis in the gonads. These sex steroids in turn act via negative or positive feedback to regulate gonadotropin production and secretion. Sex steroids modulate GnRH secretion at the hypothalamic level; however, depending on species, they may also affect *Fshb* transcription by directly targeting gonadotropes.

3.3.1 Androgens

Androgens, which include testosterone and 5α-dihydrotestosterone (DHT), directly stimulate *Fshb* expression in both rat primary pituitary cultures and in GnRH-deficient rats [199]. In LβT2 cells, androgens stimulate the murine *Fshb* promoter-reporter in a dose- and time-dependent manner when

co-transfected with the androgen receptor (AR), but not with a DNA-binding deficient form of the receptor [200]. This suggests a crucial role for AR-DNA binding to modulate *Fshb* expression. Moreover, several candidate hormone-responsive elements (HREs) exist in the proximal murine promoter, some of which are conserved across species (Figure 1.2) [201]. Androgens can also stimulate ovine and murine *Fshb* reporter activities synergistically with activins in LβT2 cells. Data obtained from rat primary pituitary cultures are, however, inconsistent with primary culture data from other species [202]. DHT treatment has no effect on FSH secretion in ovine primary pituitary cultures, and the synthetic androgen methyltrienolone (R1881) fails to increase *Fshb* mRNA levels in murine primary pituitary cultures. Nonetheless, R1881 potentiates activin A-stimulated *Fshb* mRNA expression in murine pituitary cultures, supporting a synergistic role between activins and androgens [203]. Androgens suppress both FSH release and *FSHB* mRNA expression in pituitary cultures from transgenic mice carrying a 10kb human *FSHB* minigene [204]. Similarly, androgens suppress FSH release at the pituitary level in GnRH-deficient men [205-207], casting doubt on a stimulatory role for androgens in humans. In summary, androgens regulate FSH production and release in the pituitary via species-specific mechanisms. Additional studies are required to explain the bases for these differences in mechanistic detail.

3.3.2 Estrogens

Ovarian estrogens are potent feedback regulators of FSH synthesis and secretion (see Section 1). Ovariectomized rodents, which are deficient in gonadal estrogens, exhibit increased serum FSH. FSH levels are partially suppressed with exogenous 17β-estradiol treatment [208]. The murine *Fshb* promoter does not respond to 17β-estradiol in LβT2 cells even in the presence of transfected estrogen receptor (ER)α or ERβ [200], and knockout of ERα in the pituitary has no effect on serum FSH or *Fshb* mRNA levels in females [209]. However, another study suggests that 17β-estradiol can suppress *Fshb* mRNA expression through ERα, but independent of its DNA-binding activity [210]. Although *in vivo* studies demonstrate a suppressive role for 17β-estradiol on FSH secretion, *in vitro* studies suggest that its actions are likely at the hypothalamic rather than pituitary level. Other studies suggest that additional ovarian hormones such as inhibin (Section 3.4.2) and/or progesterone (Section 3.3.3) suppress FSH at the pituitary level [211-213]. It is possible that estrogens may indirectly regulate FSH at the pituitary level as estradiol can inhibit activin A-stimulated *Fshb* mRNA expression in LβT2 cells [214]. Overall, a role for estrogens in *Fshb* transcriptional regulation in the gonadotropes is not yet clear. However, although estrogens may not regulate basal *Fshb* expression, they may have

a suppressive effect on *Fshb* expression in gonadotropes by inhibiting the stimulatory actions of activins (Section 3.4.2).

3.3.3 Progesterone

Progesterone is also a potent regulator of FSH secretion, particularly during the luteal phase of the menstrual cycle (Section 1). Progesterone (P_4) stimulates *Fshb* gene expression [215] and FSH secretion [69, 216] in rats, and induces the murine *Fshb* promoter in LβT2 cells [200]. Furthermore, antiprogestins block FSH secretion and *Fshb* mRNA expression during the secondary FSH surge in female rats [68, 217-218], suggesting that progesterone is an important factor for inducing the secondary FSH surge seen in rodents. In rat or ovine primary pituitary cultures, R5020, a synthetic progestin, can stimulate rat or ovine *Fshb* promoter-reporter activity [201, 219]. The action of R5020 on the murine *Fshb* promoter requires progesterone receptor (PR), and PR can directly bind to the murine *Fshb* promoter [200]. Several putative HREs exist in the rat and ovine *Fshb* promoters and may play a significant role in their regulation by progesterone [201, 219]. Contrary to the promoter-reporter studies, progesterone inhibits *Fshb* expression and FSH release in ovine primary pituitary cultures [202, 220]. In rat primary pituitary cultures, progestins on its own, or in collaboration with activins, stimulates FSH release and up-regulate *Fshb* mRNA levels [177, 218, 221]. The cooperative effects between progestins and activins can also be observed with the murine *Fshb* promoter-reporter in LβT2 cells [200]. Interestingly, R5020 does not affect human *FSHB* promoter-reporter activity in LβT2 cells even when co-transfected with the rat or human PR [222]. In some species, progesterone is a stimulator of *Fshb* synthesis, whereas in other species it has no effect or even acts as an inhibitor of *Fshb* synthesis. These apparently conflicting data may be attributed to the fact that the HREs in the human, ovine, and rat promoters all differ significantly (Figure 1.2). In addition, PR, a nuclear receptor, may differentially recruit co-activators versus co-repressors under different conditions to respectively activate or repress *Fshb* transcription. Whether progesterone-regulated *Fshb* expression is dependent on species-specific HREs or whether they may act through HRE independent pathways still remains to be determined.

3.4 Regulation of *Fshb* expression by TGFβ superfamily ligands

Ligands of the transforming growth factor β (TGFβ) family regulate many biological processes [223]. In a broad sense, they act to modulate growth, differentiation, and functional homeostasis of most cell types. Their roles span across a wide range of biological functions, including reproduction, the

immune response, bone formation, liver growth and regeneration, tissue remodeling and repair, erythropoiesis, and angiogenesis. TGFβ superfamily ligands include activins, inhibins, TGFβ isoforms, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDF)s, anti-Müllerian hormone (AMH), Nodal, and Lefty, and generally act as autocrine and/or paracrine factors to locally regulate cellular functions. Several TGFβ family ligands regulate *Fshb* expression. In fact, the activins and inhibins were discovered and named based on their relative effects on FSH secretion [224-227].

In the following sections, I will review TGFβ family ligand signaling in general and then specifically within the context of FSH regulation.

3.4.1 TGFβ superfamily signaling: The basics

TGFβ family ligands bind and signal through two classes of serine-threonine kinase receptors: type I and type II [223]. The ligands propagate their signals via heterotetrameric complexes of two type II receptors and two type I receptors. TGFβs and activins first bind the extracellular domains of the type II receptors, type I receptors are then recruited into the complex. In contrast, the assembly of most BMP heterotetrameric receptor complexes requires BMPs to first interact with their type I receptors. The type II receptors ACVR2 and ACVR2B are shared by activins and BMPs, whereas BMPR2 is specific for BMPs. Although sharing has been noted for type II receptors, ligand and type I receptor interactions tend to be more restricted (Figure 1.3). BMP ligands signal through type I receptors ACVRL1, ACVR1, BMPR1A, and/or BMPR1B, whereas activins signal through ACVR1B, and/or ACVR1C (for alternate receptor nomacature see Table 1.2).

Both type I and type II receptors have a short extracellular domain, a single transmembrane domain, and an intracellular serine-threonine kinase domain [223]. The intracellular domains of type I receptors are characterized by a GS (glycine and serine-rich) region located N-terminal to the kinase domain. The dimeric ligand serves as a scaffold for the assembly and stabilization of the type I and type II receptor complex; spatial proximity of the two receptor types allows the constitutively active type II receptor to *trans*-phosphorylate the GS domain of the type I receptor. Phosphorylation of the GS domain converts the type I receptor kinase to an active conformation, thus initiating downstream cellular signaling. Mutation of Thr-204 in TGFBR1, or the Gln-223 in BMPR1A to Asp or Glu results in a constitutively active type I receptor, allowing them to produce cellular responses independent of ligand and/or type II receptors [223, 228]. Activated receptor complexes can be internalized from the cell surface through two endocytic pathways: clathrin-coated vesicles or

calveolae/lipid-rafts [229-230]. Signaling can be terminated by targeting the receptor complex for degradation, or signaling can continue in endosomes [223]. For example, internalization of TGFβ receptors through the clathrin-mediated endocytic pathway promotes the colocalization of these activated receptors with their downstream signaling components and adaptor proteins [231-235]. This suggests that protein complexes can be assembled in the endocytic pathway to further elicit downstream signaling cascades.

SMADs, or mammalian homolog of drosophila mothers against decapentaplegic, are the major signal transducers of TGFβ superfamily ligands; nonetheless, the ligands can also activate SMAD-independent pathways [223]. In the SMAD-dependent pathway, the activated type I receptor kinases phosphorylate the extreme C-termini of receptor-regulated SMADs (R-SMADs) at the Ser-Ser-X-Ser (SSXS) motif, which then allows them to complex with the co-regulatory SMAD (co-SMAD) prior to accumulating in the nucleus. Together with other transcriptional activators or repressors, SMAD complexes regulate target gene expression, often through direct binding to the DNA. Eight different SMADs have been identified in mammals (Figure 1.3): SMAD1, SMAD5, and SMAD8 are BMP-specific R-SMADs; SMAD2 and SMAD3 are TGFβ/activin-specific R-SMADs; SMAD4 is the only co-SMAD; SMAD6 and SMAD7 are inhibitory SMADs (I-SMADs). However, there are exceptions regarding the specificity of R-SMAD activation. For example, though TGFβ typically activates SMADs 2 and 3 via TGFBR1, it can also stimulate phosphorylation of SMADs 1 and 5 in endothelial cells. In this case, the presence of a co-receptor, endoglin, permits recruitment of an alternate type I receptor, ACVRL1, into the complex [236-238]. Conversely, SMAD2/3 can be phosphorylated by BMP receptors in certain cell types ([239] and Ho et al. unpublished data). This area of research is still in its early stages and requires further investigation.

R-SMADs and co-SMADs contain an N-terminal Mad homology (MH1) domain and a C-terminal MH2 domain, connected by a variable linker region [223, 228, 240]. The MH1 domain mediates binding to DNA via a highly conserved β-hairpin loop [241]. The linker region of R-SMADs mainly serves as a target for post-translational modifications, such as phosphorylation, sumoylation, and ubiquitination, which determines the lifespan, function, and localization of SMAD proteins in the cell. The MH2 domain mediates interaction with receptors and oligomerization with other SMADs, whereas all three domains are capable of interacting with additional transcription factors [223, 228, 240]. The type I receptor kinase domains have a solvent exposed loop (L45 loop) between kinase subdomains IV and V [242]. R-SMADs have a corresponding structure called the L3 loop, which is a 17 amino acid region protruding from the surface of the MH2 domain [243]. It is the interaction between the L45 loop and the L3 loop that allows for the specific interaction between type I receptors

and their respective R-SMADs. The L45 loop is conserved within BMP-specific receptors and within TGFβ/activin-receptors, but differs between the two subgroups of receptors. Likewise, the L3 loop is similar within a given SMAD subgroup but differs between different SMAD subgroups [243-246]. In their unphosphorylated and inactive state, R-SMADs adopt an autoinhibitory structure where their MH1 and MH2 domains physically bind to each other to suppress each other's function. These SMAD proteins undergo a constant process of nucleocytoplasmic shuttling [247-250]. However, the majority of R-SMADs concentrate in the cytoplasm [251] due to the actions of cytoplasmic SMAD-anchors, such as the protein SARA (SMAD anchor for receptor activation) [232]. Upon phosphorylation of the SSXS motif by the type I receptor, R-SMADs undergo conformational changes that relieve them from their inhibitory conformation and thus become activated. In their active state, R-SMADs' affinity for cytoplasmic anchors decreases [252]; they expose their nuclear localization to increase their affinity for nuclear import machinery [253-254], favoring their relocation to the nucleus. In their active state, the affinity of R-SMADs for SMAD4 is also increased, allowing R-SMADs to oligomerize with SMAD4 and regulate target gene expression [255]. Dephosphorylation of R-SMADs is one event that contributes to the termination of the signal, allowing them to recycle back to the cytoplasm [249]. Conversely, R-SMAD signaling can be terminated via ubiquitin-dependent proteolysis [256].

I-SMADs, SMAD6 and SMAD7, are antagonists of R-SMAD signaling. SMAD7 inhibits both TGFβ/activin and BMP signaling [257-258], whereas SMAD6 is more selective at inhibiting BMP signaling [259-260]. Inhibitory actions of I-SMADs are diverse; they can compete with R-SMADs for interaction with activated type I receptors [257-258, 261], they can compete with R-SMADs for oligomerization with SMAD4 to form inactive complexes [260], they can bind SMAD-responsive elements disrupting R-SMAD/co-SMAD binding and recruiting co-repressors to the promoter [262-263], and they can recruit E3 ubiquitin ligases to type I receptors resulting in their ubiquitination and degradation [264-266]. Expression of I-SMAD is induced by TGFβ/activins and BMPs, and thus acts as a negative feedback signal to regulate TGFβ superfamily signaling [258, 267-269].

3.4.2 Regulation of *Fshb* expression by activins, inhibins, and follistatins

GnRH is an important regulator of both LH and FSH production; whereas activins, inhibins, and follistatins specifically regulate FSH synthesis [270-272]. Inhibins were first characterized for their ability to specifically suppress FSH release without affecting LH release from rat primary pituitary cultures [224, 227, 270]. In the process of purifying inhibins, activins were also characterized for

their stimulatory effect on FSH release [224-227]. Inhibins and activins are structurally related and both are disulfide-linked dimers. Inhibins are heterodimers of an α-subunit and one of two β-subunits (β_A and β_B), resulting in inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$). Activins, on the other hand, are homo- or heterodimers of the two inhibin β-subunits giving rise to activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$), and activin AB ($\beta_A\beta_B$) [273-274]. Later studies isolated three additional β-subunits, β_C, β_D (Xenopus only), and β_E, the subunits required for the formation of the homodimers activin C, activin D, and activin E [275-277]. Although these latter dimers have no effect on FSH secretion, the β_C subunit can form heterodimers with β_A or β_B but not the α-subunit, giving it the potential to modify the bioavailability of activins [278]. Follistatins were later discovered as single chain polypeptides that that bind to and bioneutralize activins, and thereby possess FSH-suppressing activity [279-280].

Numerous *in vivo* and cell culture studies confirmed the stimulatory effect of activins on FSH release. Injection of purified activin A increases circulating levels of FSH in both male and female rats [10-11]. Similarly, treatment of rat primary pituitary cultures with purified activin A stimulates *Fshb* expression [12] and FSH secretion [13]. As in most other tissues, activins are produced locally and act in an autocrine/paracrine fashion to target gonadotropes and other cell types in the anterior pituitary [272]. Activins also exist in the circulation, but the majority is bound to follistatins, and thus activins likely do not act as endocrine regulators of FSH [281]. Activin A, B, and AB are all expressed in the pituitary, but only activin B is expressed in rat gonadotropes and LβT2 cells [282-283]. Therefore, it was initially suggested that activin B may be the more relevant form for regulating FSH synthesis [170, 272, 283]. Nonetheless, activin A and AB are expressed by other cell types of the pituitary and may exert their effect in a paracrine fashion. This notion was challenged by a study showing that neutralizing monoclonal antibodies against the activin β_B subunit selectively inhibit *Fshb* mRNA accumulation and FSH secretion from rat anterior pituitary cultures [284-285]. These results suggest that gonadotropes endogenously express a physiological activin B tone to support basal FSH synthesis and release. Similarly, basal *Fshb* expression is maintained in LβT2 cells by endogenous activins [203, 282, 286-287]. The immunoneutralization results also suggest that activin B serves as an autocrine signal to regulate *Fshb* expression and its loss cannot be compensated by activin A. However, the deletion of the *Inhbb* gene in mice, which leads to the loss of activin B and AB, slightly increased FSH levels [288], whereas deletion of the *Acvr2*, the activin type II receptor, dramatically decreased FSH synthesis and release [289-290]. This suggests that activin A from surrounding cells, or other ligands that may act through ACVR2, can compensate for the loss of activin B and AB. Despite the conflicting data regarding the physiologically relevant activin subtype,

current studies support an important role for activin signaling in maintaining normal FSH synthesis and secretion.

3.4.2.1 Activin regulation of *Fshb* expression

Both activin type II receptors, ACVR2 and ACVR2B, are expressed in LβT2 cells; but only the deletion of *Acvr2* reduces FSH levels *in vivo*, suggesting that ACVR2 is the preferred type II receptor by which activins regulate *Fshb* expression [170, 282, 291-292]. Knockdown of *Acvr1b* specifically inhibits activin A signaling in LβT2 cells [292], suggesting that, at least *in vitro*, ACVR1B is the preferred type I receptor mediating the activin A response on *Fshb* synthesis. Further analysis suggests that in addition to ACVR1B, activin B and activin AB may also signal through ACVR1C to regulate *Fshb* transcription in LβT2 cells [293]. Mouse studies suggest that these type I receptors have distinct roles in embryonic development; while ACVR1B-deficient mice die early *in utero* [294], ACVR1C-deficient mice are viable and have no defects in fertility [295], indicating that ACVR1C does not play a significant role in activin signaling.

The mechanisms by which activins stimulate *Fshb* expression in gonadotropes also vary between species. While activin A only weakly stimulates the human *FSHB* promoter in LβT2 cells [296], it is a strong stimulator of the murine, rat, ovine, and porcine *Fshb* promoters [286, 297-300]. Activin A treatment increases SMAD2/3 phosphorylation, and causes its nuclear accumulation in LβT2 cells. This increase in SMAD2/3 phosphorylation correlates with an increase in *Fshb* mRNA levels [297, 301]. Although activin-mediated transcriptional regulation of murine and porcine *Fshb* is dependent on both SMAD3 and SMAD2, only SMAD3 is necessary for activin to stimulate the rat *Fshb* promoter [293, 296-297, 300-303], indicating that mechanistic differences between species may exist. Activated SMAD2/3 form oligomers with SMAD4 and accumulate in the nucleus to modify target gene expression. A necessary role for SMAD4 in activin A induction of *Fshb* transcription in LβT2 cells has recently been established [304].

The MH1 domain of SMAD3 mediates binding to the SMAD binding element (SBE). Initially, the SBE was thought to be composed of an 8 base pair palindromic DNA sequence, GTCTAGAC. However, it was later found that just the first half of the palindromic sequence, GTCT (or its reverse complement AGAC), is sufficient as an SBE [250, 305]. The rat and murine *Fshb* promoters contain the full length 8 base pair sequence in their activin sensitive regions and SMAD2/3/4 can bind to this element *in vitro* [296, 300, 302]. Consistently, mutations to this SBE, thus blocking SMAD binding, lead to a decrease in activin A or SMAD2/3/4 induction of the murine and rat *Fshb* promoters. Since

SMAD2 lacks DNA binding ability, activin-mediated *Fshb* transcription is dependent on the direct binding of SMAD3/4 to the promoter [286, 296, 300, 302]. In summary, activin stimulation leads to SMAD2/3/4 accumulation in the nucleus, allowing for its association with the SBE, and stimulation of murine or rat *Fshb* gene expression.

Interestingly, the SBE found in the rodent *Fshb* promoters is not conserved in the human, porcine or ovine *Fshb* promoters (see Figure 1.2). Activins weakly stimulate the human promoter, and addition of this SBE potentiates the activin response but not to levels comparable to those of the murine promoter [296]. Mutation of the SBE in the murine *Fshb* decreases activin induction only by about 50% [296], but the porcine and ovine *Fshb* promoters are sensitive to activin stimulation even without this consensus SBE [170, 299]. These data suggest the involvement of additional or different SBEs that may serve in the regulation of *Fshb* transcription. Several SBE half sites exist in the ovine promoter; mutations to two of these sites (SBE-like 1 and SBE-like 2) (Figure 1.2) greatly attenuate the activin A response [222, 298, 306]. Both of these sites are conserved across species, save for mice which have the SBE-like 1 site and the consensus full-length SBE. Mutation of either SBE-like 1 or SBE-like 2 in the porcine promoter greatly attenuates activin A stimulation. This effect is similarly observed through mutation of the SBE-like 1 and full length SBE sites in the murine promoter [286, 298, 303]. *In vitro* binding assays suggest that SMAD2/3/4 can bind to these SBE-like sites [286, 296, 298]. Although definitive binding data in any species are still lacking, data acquired thus far suggest that the two SBE-like sites are necessary to confer activin responsiveness in *Fshb* transcriptional regulation. Nonetheless, these sites alone appear to be insufficient for activin responsiveness, both sites are conserved in the human *FSHB* promoter despite negligible activin responsiveness.

3.4.2.2 SMAD-interacting proteins involved in *Fshb* regulation

SMADs bind to SBEs with low-affinity [222, 241]. To increase their binding affinity and specificity to their target genes, SMADs often depend on cooperation with protein binding partners. *Paired*-like homeodomain transcription factors 1 (PITX1) and PITX2 are known regulators of basal *Fshb* expression in gonadotropes, and appear to be important in activin A-regulated *Fshb* transcription in LβT2 cells as well. PITX1/2 can physically interact with SMAD2/3/4 [174, 307-308], and a conserved PITX binding site exists in the proximal murine *Fshb* promoter (Figure 1.2). Mutations to this site abrogate activin A and SMAD2/3/4 induction of the *Fshb* reporter in LβT2 cells [307, 309], and severely impair ovine *Fshb* promoter-reporter activity in pituitaries of transgenic mice [310]. Furthermore, depletion of endogenous PITX1/2 inhibits basal and activin A-stimulated activity of the

murine and rat reporters [307-308]. Collectively, these data suggest that PITX proteins are involved in promoting basal and activin-mediated *Fshb* transcription, possibly by helping SMAD2/3/4 complexes bind SBEs on the *Fshb* promoter.

The forkhead transcription factor FOXL2 is another transcription factor involved in *Fshb* transcriptional regulation. In the pituitary, FOXL2 is exclusively expressed in the gonadotropes and thyrotropes. FOXL2 physically interacts with SMAD3, only weakly with SMAD2, and not with SMAD4 [311-312]. A FOXL2-binding site was first identified in the porcine *Fshb* promoter juxtaposed to the SBE-like 1 element. Mutations to this binding site or depletion of endogenous FOXL2 protein dramatically inhibit activin-stimulated porcine *Fshb* promoter-reporter activity in LβT2 cells [303]. Furthermore, binding of SMAD3 and 4 to the porcine promoter can be detected when expressed together with FOXL2 [312], suggesting that FOXL2 can stabilize SMAD3/4 binding to the adjacent SBE. A single base pair difference exists in the FOXL2 binding site of the human and ovine promoters, suggesting that the promoters in these species would bind to FOXL2 with a lower affinity. Nevertheless, mutations made to this putative element in the ovine reporter can still inhibit the activin A response [306]. Therefore, although FOXL2 may bind to the putative FOXL2 binding site with a lower affinity, this site appears necessary for activin to induce *oFshb* promoter-reporter activity. Corpuz *et al.* recently showed that the FOXL2 binding site is also critical for the induction of human *FSHB* transcription by activins [313]. Whereas the human *FSHB* promoter is not responsive to SMADs, it is, nonetheless, weakly stimulated by activin A [296, 313]. FOXL2 binds to this site in the human promoter, and the slight activin responsiveness detected in the human *FSHB* promoter is dependent this FOXL2 binding site [313]. The murine *Fshb* promoter contains a two base pair difference in the FOXL2 binding site (Figure 1.2), which can only very weakly bind FOXL2 [313]. However, an additional FOXL2-binding site exists in the more proximal promoter. This second FOXL2 binding site is juxtaposed to the SBE-like 2 element (Figure 1.2), and is conserved across species. Mutations made to the second FOXL2 binding site or depletion of FOXL2 in LβT2 cells greatly reduce activin A induction of murine *Fshb* promoter activity [303]. In summary, FOXL2 or FOXL2 binding sites are required for activin-induction of the ovine, porcine, murine, and human *Fshb*/*FSHB* gene. The relevant SBE-like sequences are flanked closely by FOXL2 binding sites, and FOXL2 is capable of physically interacting with SMAD3 to stabilize its binding to the SBE-like sites on the *Fshb* promoter.

3.4.2.3 Activin and GnRH synergy in *Fshb* regulation

As previously discussed, activins act mainly through SMAD2/3 to regulate *Fshb* transcription (Section 3.4.2.1) whereas GnRH acts, at least partially, through AP1 proteins (Section 3.2). Activins and GnRH can also act synergistically to induce murine *Fshb* gene expression in LβT2 cells, and this synergy likely occurs at the level of SMAD3 and AP1. Mutations made to the full length SBE site on the murine *Fshb* promoter attenuated the independent activin A response; similarly, mutations made to the AP1 half site also inhibited the independent GnRH response, but neither mutation blocked the synergistic actions of the ligands. Only when the SBE and AP1 half site were mutated together was the synergy abrogated [192]. How SMAD3 and AP1 act together at the murine *Fshb* promoter is still not clear. Activin A enhanced GnRH-mediated phosphorylation of p38 and overall c-Fos levels, although activin A by itself had no effect on these proteins. Similarly GnRH, which does not stimulate SMAD3 phosphorylation independently, potentiated activin A-mediated SMAD3 phosphorylation [192, 314]. Therefore, the two ligands may potentiate each other's signaling by favoring the activity of their downstream effectors. As previously discussed, activins only weakly stimulate human *FSHB* promoter activity in LβT2 cells. Activin A can, nonetheless, synergize with GnRH in a SMAD2/3 and AP1 dependent manner to increase *FSHB* transcription [193]. This supports a role for activin in regulating *FSHB* gene expression in humans, albeit indirectly.

3.4.2.4 Activin regulation of *Fshb* by a SMAD-independent pathway

Although a lot of importance has been placed on the SMAD-dependent pathway for activin-mediated *Fshb* regulation, SMAD-independent pathways may also play a significant role. Inhibiting ACVR1B activity in LβT2 cells completely blocks activin's effect on the murine, rat, and porcine *Fshb* reporter activity. However, knocking down SMAD2 or SMAD3 does not completely block the activin effect [296-297, 301, 303], suggesting the possibility for a SMAD-independent pathway in the regulation of *Fshb* transcription. In comparison to the murine, rat, and porcine *Fshb* promoters, SMAD3 does not seem to play a significant role in regulation of the ovine promoter [315], which prompted investigators to probe for SMAD-independent mechanisms that may regulate ovine *Fshb* transcription. TGFβ-activated kinase 1 (TAK1) is a MAP kinase kinase kinase involved in the activation of the MAPKK6/MKK3 and p38/MPK2 signaling cascades [316-317]. Over-expression of TAK1 stimulates ovine *Fshb* promoter activity to a level comparable to that with activin treatment. In addition, inhibiting the action of endogenous TAK1 by introducing a dominant negative TAK1 construct decreases activin-mediated induction of the ovine *Fshb* reporter. Furthermore, a small

molecule inhibitor for TAK1 (5Z-7oxozeanol) completely abolishes activin A-induced *Fshb* reporter activity. This inhibitor also blocks activin A from increasing endogenous *Fshb* mRNA levels in LβT2 cells, suggesting a necessary role for the TAK1 pathway in activin-mediated induction of both ovine and murine *Fshb* transcription [315]. The mechanism by which TAK1 modulates activin-mediated *Fshb* transcription still requires further investigation.

3.4.2.5 Antagonists of activin signaling

Inhibins are structurally related to activins; however, unlike activins they act to inhibit FSH secretion [270-272]. FSH stimulates inhibin α-subunit production in granulosa/Sertoli cells, thus favoring the assembly of inhibins rather than activins in the gonads. This mode of action provides a negative feedback loop to inhibit FSH synthesis [318]. Although both inhibin α- and β-subunit mRNAs are expressed in the anterior pituitary, endocrine feedback from the gonads is likely the primary mechanism by which inhibins regulate FSH production [318-319]. Inhibins suppress FSH expression in numerous animal and cell culture studies. Injection of purified inhibin A greatly suppresses circulating FSH levels in female rats [21-26]. Conversely, injection with purified activin A increases circulating FSH [10-11], but the stimulatory effect of activins is antagonized when injected together with inhibins [11]. In contrast, neutralizing antibodies against the inhibin-specific α-subunit increase circulating FSH levels [212, 320-321]. In primary pituitary cell cultures as well, inhibin A suppresses FSH synthesis and secretion [322-323]. Differential expression of inhibin isoforms has been observed. Inhibin B is the main circulating isoform in men and male rats, whereas females express both inhibin A and B. A recent study suggests that in rats, inhibin B acts as a more potent suppressor of *Fshb* expression as compared to inhibin A [324].

As mentioned above, inhibin heterodimers share a common β-subunit with activins [9, 318]; hence inhibins can also bind to activin type II receptors. Both ACVR2 and ACVR2B can bind inhibins directly, although with a much lower affinity than with activins [325-328]. Inhibins, having only one β-subunit, are unable to assemble the complete heteromeric activin receptor complex, and thus cannot activate an activin-like signaling cascade [223, 329]. Inhibins have no signaling properties themselves; instead they act as competitive antagonists of activin receptors, and therefore prevent the formation of functional activin receptor signaling complexes. Although inhibin has an affinity 10 times lower for type II receptors as compared to activin, equimolar of inhibin is sufficient to compete for binding with activin in primary gonadotrope cultures. It was found that a co-receptor, betaglycan (TGFBR3), increases inhibin's affinity for the type II receptor, thereby increasing its potency as an

activin receptor antagonist and decreasing its IC_{50} [222-223, 330-331]. Betaglycan can bind inhibin with high affinity independent of type II receptors, but does not bind activin. Collectively, the accepted model of inhibin-mediated activin antagonism involves inhibin interacting with both betaglycan and activin type II receptors forming a stable complex, thus sequestering the activin receptors away from activin.

In contrast to inhibin, follistatins are structurally distinct from activins. They antagonize activin signaling by physically binding to activins, blocking their access to activin receptors [332-333]. Recently, the crystal structure of follistatin-288 bound to activin was determined, revealing that follistatin masks activin's binding sites for both type I and type II receptors [334]. Follistatin also promotes internalization and degradation of activins [335-336]; with less activins available, FSH synthesis and secretion are reduced [337]. In addition to sequestering activins in the bloodstream, follistatins also act as autocrine or paracrine modulators of activin signaling in the pituitary [222, 272, 319]. *Fst* mRNA is most abundantly expressed in gonadotropes [338-341] and folliculostellate cells [338-341], a cell type in the pituitary which does not produce hormones. Follistatin secreted by gonadotropes acts in an autocrine fashion to suppress FSH secretion [338-341], whereas follistatin produced by folliculostellate cells acts as a paracrine modulator of FSH secretion [342-343]. Two major follistatin isoforms exist due to alternatively spliced mRNAs (FS315 and FS288) [344-345]. A third isoform, FS303, has been recently identified in porcine follicular fluids [345]; this isoform results from a proteolytic cleavage of FS315. FS315 and FS288 differ in their abilities to associate with cell-surface proteoglycans. FS315 is the circulating form, whereas FS288 is believed to act locally due to its greater affinity for cell-surface proteoglycans [319, 345-346]. Activins are potent inducers of follistatin expression in the pituitary, which is consistent with the maintenance of homeostasis via a negative feedback mechanism [340]. Activins can act on gonadotropes to regulate follistatin production, but surprisingly, activin has no effect on *Fst* mRNA levels in folliculostellate cells, which also express activin receptors [343]. This suggests that folliculostellate cells may provide a local follistatin tone in the pituitary to control basal FSH secretion [9].

Inhibitory SMAD, SMAD7, is a negative regulator of TGFβ/activin signaling. *Smad7* is a direct gene target of ligand-activated SMAD3/4 complexes [347], and acts to antagonize activin-mediated *Fshb* expression in the gonadotrope cells [297]. Activin A rapidly stimulates *Smad7* mRNA expression in rat anterior pituitary cultures and in LβT2 cells [348]. SMAD7 competes with SMAD2/3 for association with ACVR1B thereby preventing SMAD2/3 phosphorylation and activation in murine gonadotropes [297]. Furthermore, over-expression of SMAD7 blocks activin-stimulated *Fshb* transcription [175, 297]. SMAD7 also inhibits activin signaling by other mechanisms (Section 3.4.1);

however, whether these mechanisms are relevant in the context of activin-mediated *Fshb* transcription in gonadotrope cells remains to be determined. Nonetheless, SMAD7 appears to be involved in *Fshb* transcriptional regulation by providing another negative feedback mechanism on activin signaling in murine gonadotropes.

The pharmacological inhibitor, SB431542, is commonly used to study activin signaling. It is a small molecule inhibitor that specifically targets the type I receptors ACVR1B, TGFBR1 and ACVR1C, hence TGFβ and activin signaling [349]. SB431542 is a potent inhibitor of type I receptor mediated SMAD2/3 phosphorylation and basal/activin-stimulated *Fshb* expression in LβT2 cells [282]. SB431542 acts as a competitive antagonist at the ATP binding site of the highly related ACVR1B, TGFBR1 and ACVR1C kinase domains. The kinase domains of these three type I receptors are more than 82% identical to each other, whereas the kinase domains of ACVR1, BMPR1A, and BMPR1B are all less than 68% identical to that of TGFBR1. Due to such differences, SB431542 is unable to alter BMP induced SMAD1/5/8 phosphorylation ([349], unpublished data).

3.4.3 Regulation of *Fshb* expression by bone morphogenetic proteins

3.4.3.1 BMP ligands, receptors, and signaling

Originally identified as inducers of ectopic bone and cartilage formation [350], bone morphogenetic proteins (BMPs) are now known to play important roles in cell differentiation, organ development, morphogenesis, and homeostasis [223]. BMPs are part of the TGFβ superfamily, and over 20 BMP members have been characterized. BMP ligands can be further classified into several subgroups based on their structural homology; for example BMP2 and BMP4 form one subgroup, and BMP5, BMP6, BMP7, and BMP8 form another (for the different subgroups of BMPs, see Table 1.3) [223, 228]. BMPs exist as either homodimers or heterodimers. Each monomer has six highly conserved cysteine residues that form three intramolecular disulfide bonds, termed the cystine knot motif, to stabilize the protein structure [240, 351-353]. A seventh conserved cysteine forms the disulfide bond between monomers covalently linking them together [240, 351]. Heterodimers of BMP2/5, BMP2/6, and BMP2/7 have been observed, and these heterodimers act more potently than their respective homodimers in certain contexts [354-355]. Crystal structures suggest that BMP dimers have an overall “wrist and knuckle” or “two banana” structure (Figure 1.4) [223, 356-359]. Two receptor binding sites have been identified in the BMP2 dimer; the wrist epitope is a high-affinity binding site

for the type I receptor BMPR1A, whereas the knuckle epitope is a lower-affinity binding site for the type II receptors, ACVR2, ACVR2B, and BMPR2 [228, 360-362].

BMPs typically signal through type I receptors ACVRL1, ACVR1, BMPR1A, and/or BMPR1B, and type II receptors ACVR2, ACVR2B, and/or BMPR2. The structures of BMPR1A and BMPR1B are very similar, as are the structures of ACVRL1 and ACVR1. ACVR1 and BMPR1A are widely expressed in various cell types, whereas BMPR1B expression is restricted to certain cell types. ACVRL1 is mainly expressed in endothelial cells [223, 228]. BMPs bind to type I receptors with varying affinities. BMP2 and BMP4 bind to BMPR1A and BMPR1B with a higher affinity than to other type I receptors [363], whereas BMP6 and BMP7 bind preferentially to ACVR1 but also bind weakly to BMPR1B [364]. GDF5 only binds to BMPR1B [365], and BMP9 and BMP10 preferentially bind to ACVRL1 and ACVR1 [357, 366-367]. As for type II receptors, BMP6 and BMP7 preferentially signal through ACVR2. On the other hand, BMP2 and BMP4 preferentially signal through BMPR2, but can use ACVR2 or ACVR2B in its absence [228, 368]. BMPR2 is different from other type II receptors in that it has short and long isoforms (BMPR2-S and BMPR2-L). BMPR2-L is expressed in most cell types, whereas BMPR2-S is more limited in its expression [228, 369]. For example, the long isoform is detectable in gonadotropes, but the short form is not (Rejon, Pertchenko, and Bernard, unpublished data). BMPR2-L uniquely has a long C-terminal tail following the kinase domain which may determine BMP signaling specificity, as well as act as a scaffold allowing for interaction with adaptor proteins and assembly of different signaling complexes [240]. BMPs may bind the high affinity type I receptor complex first, upon which the type II receptors are recruited to the complex forming the BMP-induced signaling complex (BISC) [240]. However, BMPs can also bind to preformed complexes (PFC) [370-371]. It was suggested that these different receptor complexes can activate distinct signaling pathways. BISC activates SMAD-independent signaling pathways, whereas PFC activates SMAD-dependent signaling pathways [230].

The SMAD-dependent pathway is by far the most thoroughly characterized BMP signaling pathway. BMP typically activates the R-SMADs, SMADs 1, 5, and 8, via C-tail phosphorylation [228]. The overall structure of SMAD1, 5, and 8 are highly similar; however, the primary structure of SMAD1 and SMAD5 are more similar to each other than to SMAD8 [372]. Conditional knockouts of SMAD1, 5, and 8 in the somatic cells of the ovaries and testes suggest significant functional redundancy between SMAD1 and SMAD5 but not with SMAD8 [373]. Like SMADs 2 and 3, activated SMAD1, 5, and 8 complex with SMAD4 to regulate gene transcription [250, 374-375]. The MH1 domain of SMAD1/5/8 mediates binding to GC-rich BMP response elements (BRE) in target

genes [269]. However, recent X-ray crystallography studies suggest that SMAD1 may also bind to SMAD3/4 SBEs (GTCT or AGAC, see Section 3.4.2.1 above) with high affinity using the analogous β-hairpin loop to that seen in SMAD3, challenging the original notion that BMPs specifically bind GC-rich motifs [376].

Nevertheless, several GC-rich BREs have been described in BMP responsive promoters. These BREs include the GCCGnCGC motif found in the promoters of *Smad6* and inhibitor of DNA binding 1 (*Id1*) [269, 377], and the TGGCGCC sequence found in the promoters of *Smad7* and *Id3* [378-379]. BMPs stimulate the expression of a variety of target genes depending on the cellular context, but I-SMADs [258, 269, 380] and Id proteins [381-388] are two general targets of BMP signaling. Id proteins mainly act to inhibit the action of basic helix-loop-helix (bHLH) transcription factors [381, 388]. Inhibition of bHLH transcription factors is often associated with inhibition of cell differentiation [384, 387]. BMPs induce *Id1* expression in a number of cell types, making it a convenient marker for BMP activity [223].

3.4.3.2 A role for BMPs in the pituitary

Several observations support a role for BMPs in the gonadotrope. BMP2 and BMP4 are involved in murine pituitary organogenesis and cellular differentiation [389]. BMP4 is required for the formation of the Rathke's pouch rudiment, an embryonic structure that gives rise to the anterior pituitary [390], whereas BMP2 acting with fibroblast growth factor 8 (FGF8) in opposing gradients determines gonadotrope cell differentiation [391]. Roles for BMPs as autocrine/paracrine regulator of pituitary and gonadotrope function in adulthood have only recently become the subjects of more intensive investigation. Repulsive Guidance Molecule b (RGMb), a BMP co-receptor, is expressed in the pituitary; interestingly, its expression overlaps with that of FSH [392], suggesting that BMPs and the associated RGMb may have a role in *Fshb* regulation in gonadotropes.

Until recently, activins were thought to be the only TGFβ family ligands that stimulate *Fshb* expression. The administration of inhibin or follistatin to murine primary pituitary cultures or LβT2 cells inhibits both *Fshb* mRNA levels and FSH secretion [393-394]. Their inhibitory effects were initially attributed to their antagonizing effects on activins; however, recent studies demonstrate that inhibins and some forms of follistatin can also antagonize BMP signaling [395-398]. Inhibins can bind to ACVR2, ACVR2B, and with the help of betaglycan, can also bind BMPR2, thus permitting inhibins to compete with BMPs for binding to all three BMP type II receptors [223, 399]. Follistatin

was shown to bind BMP7 and antagonize its effects in early *Xenopus* embryo development and in mink lung epithelial (Mv1Lu) cells [397, 400]. Collectively, the data suggest that the inhibitory effects of inhibins and follistatin on FSH synthesis might be due to the neutralization of BMPs as well as activins.

3.4.3.3 BMPs can regulate *Fshb* expression

Recently, Huang et al. developed a transgenic pituitary system where pituitary cultures were derived from o*Fshb*-luc transgenic mice. These mice express the luciferase reporter gene under the control of approximately 4 kb of the ovine *Fshb* promoter, restricting their expression only to gonadotropes [401]. Pituitary cultures from these mice were treated with activin A, BMP6, BMP7, and TGFβ1. As predicted, activin A treatment increased o*Fshb*-luc transgene activity, whereas TGFβ1 had no effect [402]. Unexpectedly (at the time), BMP6 and BMP7 also stimulated transgene activity, though their effects were not as potent as those of the activins. BMP6 and BMP7 could similarly induce *oFshb* reporter activity in transiently transfected LβT2 cells [401]. This was unanticipated because BMPs and activins were previously shown to antagonize each other's activity in some contexts. For example, activins induce dorsalization in developing *Xenopus* embryos, whereas BMPs induce ventralization [403-404]. Moreover, activin-mediated and BMP-mediated R-SMADs compete for oligomerization with the same co-SMAD (SMAD4). Huang et al. further demonstrated that endogenous BMPs may play an important role in sustaining endogenous *Fshb* expression. BMP7 bio-neutralizing antibodies decreased basal luciferase expression by 88% in transgenic murine pituitary cultures, compared to a 95% reduction with follistatin. Anti-BMP7 also decreased FSH secretion by 48%, compared to 34% repression with follistatin. This was similarly seen in rat and sheep pituitary cultures where anti-BMP7 also reduced FSH secretion by 40-56% [401]. A later study showed that BMP15 selectively and more potently stimulated *Fshb* transcription in LβT2 cells [405]. However, it is not clear whether BMP15 acts as an endogenous regulator of FSH since its expression in LβT2 cells and murine pituitary cells is very low. *Bmp15* mRNA can neither be detected in human or ovine pituitaries [406-408]. In contrast, BMP2 and BMP4, which are readily detectible in murine pituitaries, also stimulated *Fshb* transcription in LβT2 cells. In fact, their stimulatory effect was 10-fold more potent than BMP6 or BMP7 [282]. These studies demonstrated that gonadotropes have a fully functional BMP signaling system and BMPs may play a role in regulating *Fshb* expression.

Both LβT2 cells and murine pituitaries express *Bmp6*, *Bmp7*, and *Bmp8b*. However, *BMP8a* is only detected in LβT2 cells, and *Bmp2, 3, 4, 5,* and *Gdf10* are only detected in whole murine pituitaries

[282, 401, 405, 409]. Thus, depending on the subtype, BMPs may act as an autocrine or paracrine regulators of *Fshb* synthesis. All the required BMP type I receptors, type II receptors, and R-SMADs are expressed in both LβT2 cells and murine pituitaries [282, 401, 409]. Although BMPs and BMP receptors are also detected in ewe pituitaries, BMP4 and BMP6 selectively inhibit FSH secretion in ovine primary pituitary cultures [408]. BMP4 inhibits FSH release and *Fshb* mRNA expression by 40% without affecting LH release or *Lhb* mRNA expression. SMAD1 phosphorylation increases with BMP4 treatment, suggesting a functional BMP signaling cascade. However, it is not known whether the inhibitory effect of BMP4 on *Fshb* expression is dependent on SMAD1 activation. Furthermore, BMP4 antagonizes the stimulatory effects of activin A and enhances the inhibitory effects of 17β-estradiol on FSH release and *Fshb* mRNA expression in ewe pituitary cells [408, 410].

The discrepancy between the rodent and the ewe experiments may be due to several reasons. For example, there may be differences in the intracellular milieu between LβT2 cells and ovine gonadotrope cells in mixed cultures. The expression patterns of BMPs and BMP receptors are different in mice than in ewes. For example, *Bmp6*, *Bmp15*, and *Bmpr1b* are expressed in murine pituitaries, whereas *Bmp6* and *Bmp15* cannot be detected ovine pituitaries, and *Bmpr1b* is absent from ovine gonadotropes [408]. Different BMPs can and do act through different BMP receptors (as reviewed in Section 3.4.3.1), and the availability of different co-activators or co-repressors between murine and ovine gonadotropes may result in different outcomes of BMP signaling. Primary pituitary cultures are composed of a mixture of cell populations from the anterior pituitary, whereas LβT2 cells are a homogeneous transformed cell population. It is possible that BMP4 stimulates *Fshb* transcription in ovine gonadotropes while at the same time stimulating other cells in the culture to produce inhibitory signals, which act to antagonize the stimulatory effect of BMP4 on *Fshb* transcription. Nonetheless, BMPs stimulate *Fshb* expression in murine primary cultures [401]. The difference might also be explained by the concentrations of BMPs used in the different studies. One μg/ml of BMP6 or BMP7 stimulates *Fshb* transcription in murine pituitary cultures [401], whereas 25 ng/ml of BMP4 or BMP6 inhibits *Fshb* transcription in ewe pituitary cultures [408]. Perhaps, low concentrations of BMPs stimulate the expression of factors, such as follistatin from folliculostellate cells, to inhibit *Fshb* transcription, whereas higher concentrations of BMPs might stimulate gonadotropes to produce enough FSH to overcome the inhibitory signals from surrounding cells. Despite the inter-species differences, BMPs seem to play a role (inhibitor or stimulatory) in FSH regulation.

Several naturally occurring mutations found in sheep also suggest a role for BMPs in reproduction, and perhaps FSH regulation. Inverdale and Hanna sheep carry a mutation in BMP15, resulting in the

formation of a biologically inactive protein. Ewes homozygous for this mutation are sterile [406, 411-412]. On the other hand, Booroola sheep carry a missense point mutation in the intracellular serine/threonine kinase domain of BMPR1B. Homozygous Booroola mutants have a higher ovulation rate and higher circulating FSH levels than wild-type animals [411-415]. Granulosa cells and theca cells obtained from the ovaries of sheep expressing this mutant receptor show increased responsiveness to BMP2, 4, and 6 [416-417]; furthermore, differences in BMP signaling were observed between pituitary cultures from Booroola and wild-type sheep [418]. Provided that BMP signaling has an impact on FSH synthesis, the BMPR1B mutation in gonadotropes may explain, in part, the Booroola phenotype (see Chapter 2).

3.4.3.4 Activin and BMP synergy in *Fshb* regulation

Although BMP2 and 4 are less potent than activin A and B in stimulating *Fshb* expression in LβT2 cells, they can act in synergy with activins to induce *Fshb* expression [282]. BMP2 and activin A independently stimulate the murine, porcine, and ovine *Fshb* promoter-reporters, but the two ligands together synergistically promote *Fshb* reporter activity in all three species. This suggests that this synergy may be a general mechanism of *Fshb* regulation. The synergism is similarly reflected in *Fshb* mRNA levels from LβT2 cells; activin A alone elicits a 12-fold increase, BMP2 alone elicits a 2-fold increase, but together they elicit a 27-fold increase in *Fshb* mRNA expression. Interestingly, this synergism was not observed with BMP6 or 7 possibly due to their lower potencies. BMP2 and activin A likely stimulate *Fshb* transcription using two separate intracellular mechanisms as neither ligand affected the timing or magnitude of the other. The hypothesis is that activation by one ligand increases the baseline promoter activity, whereas activation by the other produces its effect dependent on the new baseline. As BMPs can potentiate the effect of activins, it is possible that exogenous BMPs may simply potentiate the effects of endogenous activin B on *Fshb* expression rather than produce their own independent effects. However, BMP2 can still stimulate the *Fshb* reporter 2-3 fold from basal when endogenous activin action is inhibited, suggesting that BMP2 can directly stimulate *Fshb* transcription as well [282].

A recent study confirmed the synergism between BMPs and activins using BMP4 and activin A. It further demonstrated that BMPs may potentiate the synergistic actions of activin A and GnRH on *Fshb* transcription [409]. Activin A treatment increases FSH secretion and *Fshb* mRNA expression in LβT2 cells. GnRH and activin A together synergistically increase FSH secretion and *Fshb* mRNA expression. Finally, BMP4 further potentiates the GnRH and activin A synergism. Although GnRH

and GnRH with activin A stimulate LH secretion and *Lhb* mRNA expression, BMP4 does not further increase their individual or combined responses. Surprisingly, BMP4 alone has no effect on FSH secretion and *Fshb* mRNA expression in LβT2 cells in these experiments. BMP4 induces SMAD1/5 phosphorylation, and activin A induces SMAD2/3 phosphorylation in LβT2 cells, but when added together, neither ligand alters the phosphorylation activity of the other. BMP4 also does not increase the activity of GnRH-mediated pathways such as ERK1/2, p38 MAPK, and pCREB. These data support the idea that BMPs, activins, and GnRH act though different, but complementary mechanisms to regulate *Fshb* transcription [409].

3.4.3.5 BMP receptors and SMADs involved in *Fshb* regulation

Over-expression experiments were used to determine the mechanism by which BMP2 may regulate *Fshb* expression [282]. The data suggested that BMP2 signals through preformed complexes of ACVR1 and BMPR2 receptors to activate SMAD-dependent pathways in gonadotropes. Although BMP2 increased the phosphorylation of all three BMP R-SMADs, over-expression data suggested that BMP2 propagates its signal through SMAD8 and SMAD4 to drive *Fshb* transcription [282]. However, these data were obtained through over-expression approaches alone; therefore they do not definitively demonstrate that these are the required receptors and SMADs in this system.

3.4.3.6 Possibility for a SMAD-independent pathway in BMP-mediated *Fshb* regulation

In addition to SMAD-dependent signaling, BMPs are also capable of initiating SMAD-independent signaling. Similar to activins, BMPs can activate JNK and p38 MAP kinase pathways [228, 240]. TAK1, previously described to be involved in activin-mediated *Fshb* transcriptional activation (see Section 3.4.2.4 above), is also activated by BMP2 and BMP4 to induce SMAD1 phosphoryation in some contexts [317, 419-421]. Furthermore, the long C-terminal tail of BMPR2 may act as a scaffold to facilitate the assembly of different SMAD-independent signaling complexes [223, 228, 240, 369]. However, a role for SMAD-independent pathways in BMP-regulated *Fshb* transcription has not yet been documented.

3.4.3.7 Antagonists of BMP signaling

BMP antagonists are secreted proteins that bind to BMPs and prevent them from binding to their specific receptors. The activity of BMP antagonists is therefore analogous to that of follistatin on activin. Such proteins include noggin, chordin, chordin-like proteins, gremlin, twisted gastrulation (Tsg), cerberus, and DAN proteins [223, 240, 422]. One of the better understood BMP antagonist is noggin, which is up-regulated by BMPs, creating a negative feedback-based antagonism [423]. Noggin binds with varying affinities to BMPs 2, 4, 6, and 7, and GDF5 [240]. Noggin-BMP7 X-ray crystal structures suggest that noggin occludes BMP7 type I and II receptor binding sites [424]. Provided that BMPs are indeed regulators of *Fshb* transcription, any of the antagonists that block the actions of BMP would be expected to inhibit *Fshb* expression in murine pituitary cultures and LβT2 cells.

A small molecule inhibitor specific for BMP receptors was recently characterized. Compound C, or dorsomorphin, was originally isolated as a small-molecule inhibitor for AMP-activated protein kinase (AMPK) [425]. Later it was discovered to also inhibit ACVR1, BMPR1A, and BMPR1B signaling and block BMP-induced SMAD1, 5, and 8 phosphorylation. Compound C has no effect on ACVR1B, TGFBR1 or ACVR1C mediated SMAD2 or 3 phosphorylation [426]. Additionally, SMAD1/5/8 inhibition can be achieved at concentrations lower than those needed to inhibit AMPK, supporting it as a BMP-receptor specific inhibitor. It was later shown that Compound C can also inhibit ACVRL1 signaling [427]. A recent report claimed that Compound C also inhibits the activation of SMAD-independent pathways, namely p38, Akt, and ERK1/2, in C2C12 cells, suggesting that this compound is effective at inhibiting both SMAD-dependent and independent pathways [428]. However, Compound C only very slightly inhibits the phosphorylation of these MAP kinases and this slight inhibition is only apparent with high concentrations of the compound. Therefore, it appears that Compound C is more effective at inhibiting the SMAD-dependent pathway than SMAD-independent pathways. The mechanism of action of this compound is not clear; however, it does not appear to act as an inhibitor of ATP binding. Derivatives of this compound are further being optimized to increase their specificity and potency for BMP type I receptors [429]. Such inhibitors will no doubt provide the means to further dissect BMP signaling in a variety of BMP regulated systems. Indeed, we have already begun to exploit this compound to study BMP regulation of *Fshb* transcription in gonadotrope cells (Chapter 2).

4. Rationale for Thesis

FSH is a crucial hormone for regulating normal fertility both in males and females. Although mutations in *FSHB* and *FSHR* in humans are rare, there are many unexplained cases of sub-fertility or infertility. Approximately one in seven couples in the UK has fertility problems, with 25% of cases being of idiopathic origin [430-432]. To date, there is no single factor that is common between all sub/infertile patients; many have no identified mutations in canonical components of the HPG axis. Because FSH expression is tightly regulated by a variety of factors, there may be defects in accessory pathways that may contribute to sub/infertility. Therefore, the characterization of such pathways is important to fully understand the regulation of FSH expression and to potentially treat sub-fertile and infertile patients.

Much effort has been made to understand the regulation of *Fshb* expression. GnRH, sex steroids, and activins are all characterized as modulators of *Fshb* transcription; however, the effects of these modulators demonstrate considerable variability between species. This implies that in different species, FSH is differentially regulated to control different ovulation patterns.

BMPs stimulate *Fshb* expression in murine pituitary cultures and LβT2 cells, both independently and synergistically with activins [282, 401, 405, 409]. Whereas the stimulatory action of activins on *Fshb* transcription has been extensively studied, the role of BMP signaling in gonadotropes, specifically in *Fshb* transcriptional regulation, is novel. Investigations of BMP actions in gonadotropes have largely been limited to the examination of gonadotropin subunit expression or gonadotropin secretion. Studies into the underlying mechanisms have been more limited, if not lacking altogether. It has been established that adult gonadotropes and LβT2 cells have a fully functional BMP signaling system [282, 401, 405, 409], suggesting a role for BMP in gonadotropes during the adult life. With the availability of current *in vitro* tools, the mechanisms through which BMP signals in gonadotropes to regulate *Fshb* and other targets are primed for analysis. The goal of my thesis is to understand the signaling mechanism by which BMP2 stimulates *Fshb* transcription in gonadotrope cells.

Figure legends

Figure 1.1: Hypothalamic-pituitary-gonadal (HPG) axis. Adapted from: https://webapp.walgreens.com/cePharmacy/programsHTML/images/Image28.gif

Figure 1.2: Alignment of proximal *Fshb/FSHB* promoters in pig, sheep, human, mouse, and rat. ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align the proximal 400 bp from the 5′ flanking regions of the *Fshb/FSHB* genes in the indicated species. Nucleotides are numbered relative to the transcription start site. Defined *cis*-elements are labelled and boxed. Nucleotides boxed in gray indicate differences from the experimentally defined *cis*-element in other species. An asterisk (*) marks the nucleotides conserved across species and gaps (−) have been introduced to facilitate the alignment.

Figure 1.3: Canonical signaling by ligands in the TGFβ superfamily. Adapted from: http://www.med.lu.se/var/plain/storage/images/media/images/bilder_labmed_lund/bilder_molekylaer_medicin_och_genterapi/smad/144046-1-eng-GB/smad_large.jpg

Figure 1.4: Crystal structure of BMP6. Generated in Pymol from PDB# 2QCW. *Allendorph, G.P. 2007. Biochemistry. 46: 12238-12247.* The two monomers of BMP6 are coloured in pink and yellow. Figure shows the wrist-knuckle/wrist-knuckle interaction with the two thumbs (the α-helices) pointing towards and away from the plane of the paper.

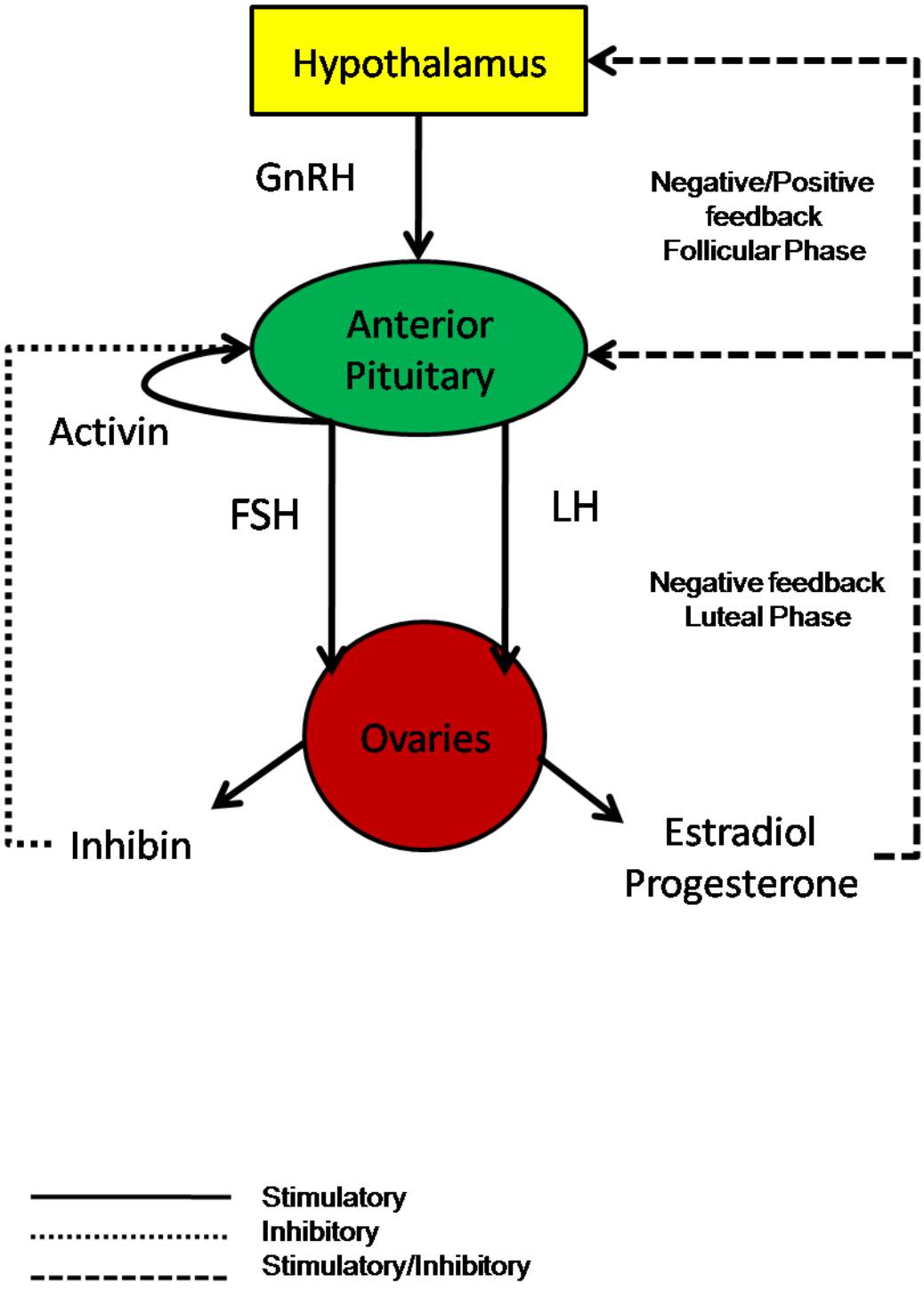
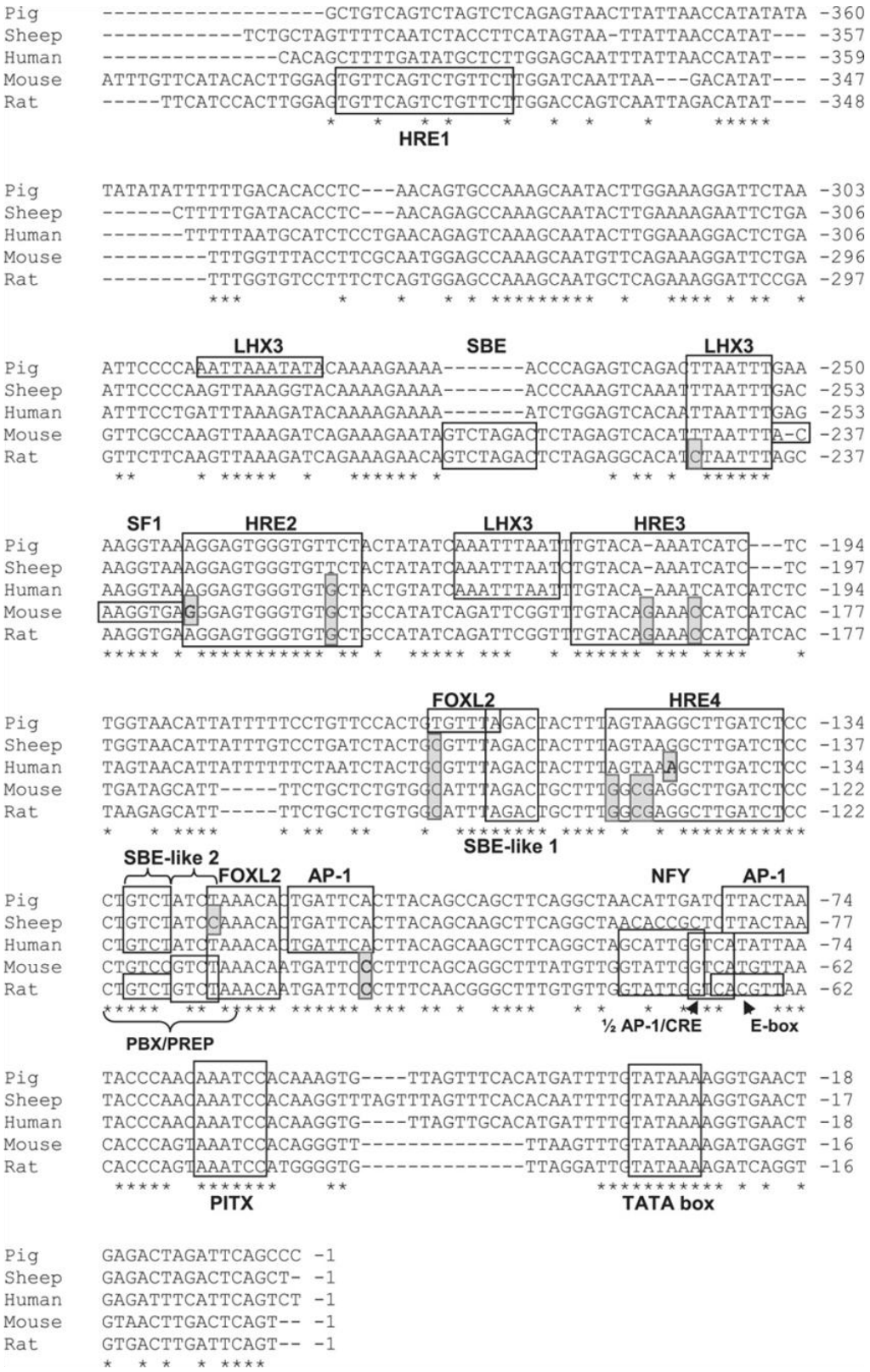

Hypothalamus
GnRH
Negative/Positive feedback Follicular Phase
Anterior Pituitary
Activin
FSH
LH
Negative feedback Luteal Phase
Ovaries
Inhibin
Estradiol
Progesterone
Stimulatory
Inhibitory
Stimulatory/Inhibitory

Figure 1.1



Bernard, D. J. 2010. Fertil Steril. 93(8):2465-85.

Figure 1.2

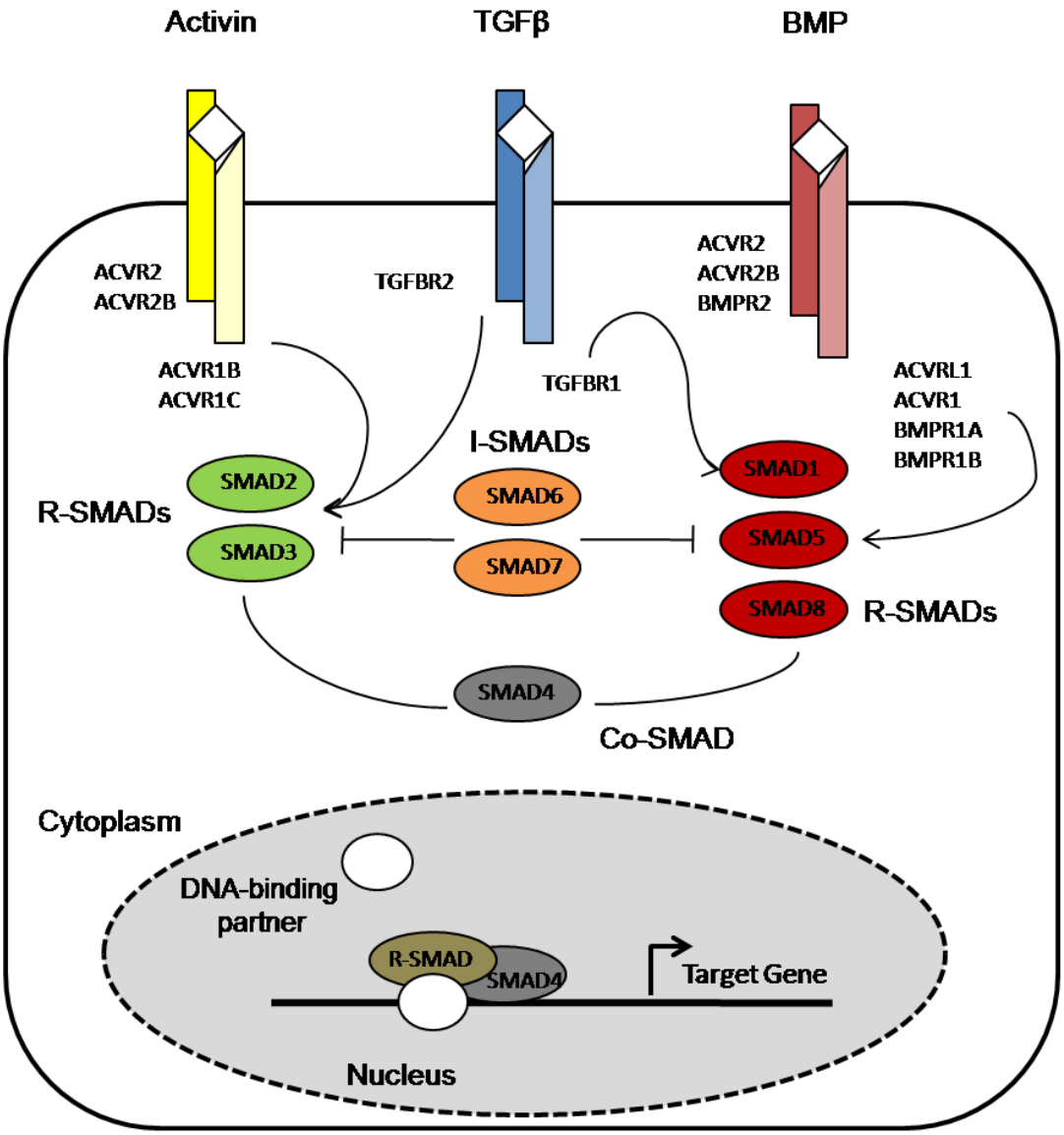

Activin
TGFβ
BMP
ACVR2
ACVR2B
TGFBR2
ACVR2
ACVR2B
BMPR2
ACVR1B
ACVR1C
TGFBR1
ACVRL1
ACVR1
BMPR1A
BMPR1B
I-SMADs
SMAD2
SMAD6
SMAD1
R-SMADs
SMAD3
SMAD7
SMAD5
SMAD8
R-SMADs
SMAD4
Co-SMAD
Cytoplasm
DNA-binding
partner
R-SMAD
SMAD4
Target Gene
Nucleus

Figure 1.3

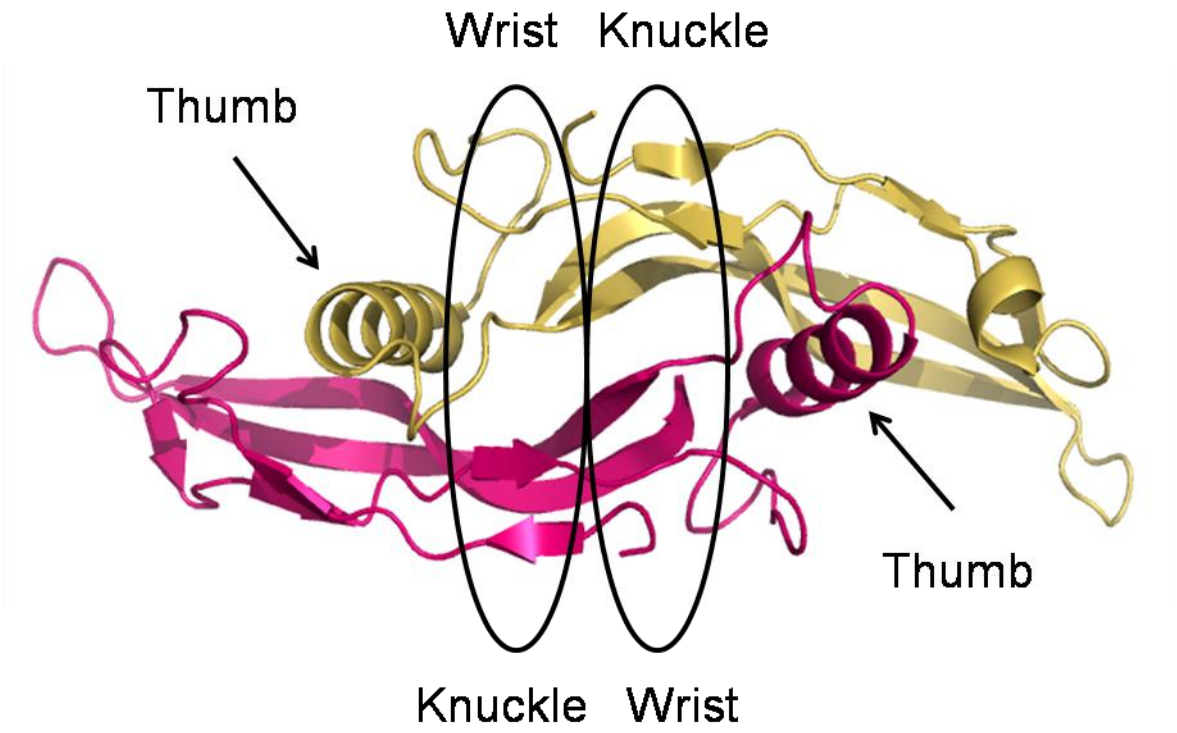


Figure 1.4

Table 1.1 Summary of mutations in the human *FSHB* and *FSHR* genes

Gender	Gene	Mutation	Characteristics	Reference
Female	*FSHB*	Val61X	Primary amenorrhea, infertility	Matthews et al. 1993
Female	*FSHB*	Cys51Gly and Val61X	Primary amenorrhea, infertility	Layman et al. 1997
Female	*FSHB*	Ala79X	Primary amenorrhea, infertility	Kottler et al. 2009
Male	*FSHB*	Cys82Arg	Azoospermia, normal puberty	Lindstedt et al. 1998
Male	*FSHB*	Val61X	Azoospermia, no pubertal development	Phillip et al. 1998
Female	*FSHR*	Ala189Val	Primary amenorrhea, developing follicles, no ovulation	Aittomaki et al. 1995
Male	*FSHR*	Ala189Val	Normal puberty, small testes, fertile	Tapanainen et al. 1997
Female	*FSHR*	Ile160Thr and Arg573Cys	Secondary amenorrhea	Beau et al. 1998

Table 1.2 TGFβ superfamily ligand receptor nomaclature

Type I receptors	
Gene Name	*Alternative name*
ACVR1L	ALK1
ACVR1	ALK2
BMPR1A	ALK3
ACVR1B	ALK4
TGFBR1	ALK5
BMPR1B	ALK6
ACVR1C	ALK7
Type II receptors	
Gene Name	*Alternative name*
ACVR2	ACTR2A
ACVR2B	ACTR2B
BMPR2	
TGFBR2	
MISR2	AMHR2

Table 1.3 BMP subgroups in vertebrates

BMP subgroups in vertebrates
BMP2 BMP4
BMP3 BMP3b
BMP5 BMP6 BMP7 BMP8 BMP8b
BMP9 BMP10
GDF5 GDF6 GDF7
BMP15 BMP9
BMP11

Modified from [223]

Chapter 2

BMPs 2 and 4 signal by first binding to specific type I receptors, followed by the recruitment of type II receptors into the signaling complex. In Chapter 2, I aimed to identify the specific type I and type II receptors through which BMP2 signals to stimulate *Fshb* transcription. Previous studies conducted in our lab used over-expression assays to assess the relative importance of the three BMP type I receptor (ACVR1, BMPR1A, and BMPR1B) in *Fshb* transcriptional regulation [282]. The data suggested a role for ACVR1, and that co-expression of the type II receptor BMPR2 was required for its stimulatory effect. However, the data from these earlier experiments required validation as the saturation of the cell membrane with both type I and type II receptors may result in the activation of signaling pathways that are normally not activated in response to ligand under physiological conditions. Furthermore, the results from over-expression experiments with the wild type versus constitutively active forms of ACVR1 and BMPR1A were inconsistent, prompting further investigation here with complementary assays. Finally, expression of BMPR1B was never examined in prior experiments in the lab. Hence its role in *Fshb* regulation remained to be determined. A role for BMPR1B is particularly interesting because a naturally occurring missense mutation in the BMPR1B kinase domain dramatically affects fertility in Booroola ewes [411-415, 433]. The effect of this mutation has been extensively studied in the ovaries [416-417], but had never been characterized in gonadotropes. Elevated plasma FSH levels in these sheep suggested that the mutation might have a stimulatory effect on FSH synthesis [433]. High FSH levels often result in the maturation and subsequent ovulation of multiple follicles, which is also a characteristic of the Booroola ewes. Recent studies have confirmed that BMPR1B is expressed in the murine pituitary and immortalized LβT2 cells [282, 409]. The Booroola mutant could be of great use in the study of BMPs as regulators of *Fshb* transcription. If this mutation has a signaling dysfunction in gonadotropes, either a gain of function or a loss of function, then it might offer some insight to the role of BMPs in *Fshb* transcriptional regulation and perhaps explain the underlying cause of the Booroola phenotype.

Title: Bone morphogenetic protein 2 signals via BMPR1A to regulate follicle-stimulating hormone beta subunit transcription

Abbreviated title: BMP2 signals through BMPR1A in gonadotropes

Authors: Catherine C. Ho and Daniel J. Bernard

Affiliation: Department of Pharmacology and Therapeutics, McGill University

Corresponding author:

Daniel J. Bernard, Ph.D.

Department of Pharmacology and Therapeutics

McGill University

3655 Promenade Sir William Osler

Montreal, Quebec (Canada)

H3G 1Y6

Ph: 514-398-2525

Fx: 514-398-6705

Email: daniel.bernard@mcgill.ca

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Abstract

Follicle-stimulating hormone beta subunit (*Fshb*) expression is regulated by transforming growth factor beta (TGFβ) superfamily ligands. Recently, we demonstrated that bone morphogenetic proteins (BMPs) stimulate *Fshb* transcription alone and in synergy with activins. Also, transfection of the BMP type II receptor, BMPR2, and constitutively active forms of the type I receptors, activin A receptor, type I (ACVR1) or BMP receptor, type IA (BMPR1A), in immortalized gonadotrope cells, LβT2, stimulated murine *Fshb* promoter-reporter activity. A third type I receptor, BMP receptor, type IB (BMPR1B), is also expressed in LβT2 cells, but we did not previously assess it functional role. A point mutation in BMPR1B (Q249R) is associated with increased ovulation rates and elevated FSH levels in Booroola (*FecB*) sheep. Here, we assessed whether BMPR1B can regulate *Fshb* transcription in LβT2 cells and whether its ability to do so is altered by the Q249R mutation. As with ACVR1 and BMPR1A, co-expression of BMPR1B with BMPR2 increased *Fshb* promoter-reporter activity in both BMP2-dependent and –independent fashion. Unexpectedly, the BMPR1B-Q249R mutant was equivalent to wild-type in its ability to stimulate SMAD1/5 phosphorylation and *Fshb* transcription. Pharmacological inhibition of ACVR1, BMPR1A, and BMPR1B confirmed that one or more of these receptors are required for BMP2-stimulated SMAD1/5 phosphorylation and *Fshb* reporter activity. Knockdown of endogenous BMPR1A, but not ACVR1 or BMPR1B, significantly impaired BMP2's synergism with activin A. Collectively, these data suggest that BMPR1A is the preferred BMP2 type I receptor in LβT2 cells and that neither ACVR1 nor BMPR1B compensates for its loss. The specific mechanism(s) through which the Booroola (*FecB*) mutation alters BMPR1B function remains to be determined.

Introduction

The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are essential reproductive hormones. Both are secreted from gonadotropes of the anterior pituitary, but the two act to regulate different aspects of gonadal function. The gonadotropins are heterodimeric glycoproteins (α/β), with their β subunits determining both rates of mature hormone synthesis and biological specificity. Both FSH and LH are regulated by gonadotropin-releasing hormone (GNRH1) secreted from the hypothalamus and gonadal sex steroids; however, endocrine/paracrine TGFβ superfamily ligands, such as activins and inhibins, act to selectively regulate FSH synthesis. Activins signal via a combination of type II (ACVR2 or ACVR2B) and type I receptors [ACVR1B and ACVR1C; also known as activin receptor-like kinase (ALK) 4 and ALK7], and downstream signaling effectors, SMADs 2 and 3, to up-regulate FSHβ subunit (*Fshb*) transcription [291, 293, 296-297, 301]. Inhibins, in contrast, suppress *Fshb* expression by blocking activins' actions through a competitive binding mechanism [434].

Recently, other members of the TGFβ superfamily, the bone morphogenetic proteins (BMPs), were shown to stimulate *Fshb* transcription alone and in synergy with activins [282, 401, 405, 409]. BMPs are expressed in LβT2 cells, an immortalized murine gonadotrope cell line, and in adult murine pituitary [282, 401, 405], and therefore might regulate FSH synthesis in vivo. We previously reported that BMPs 6 and 7, although endogenously expressed in LβT2 cells, only modestly regulate *Fshb* transcription. In contrast, BMPs 2 and 4 stimulate *Fshb* transcription more potently, but their expression in LβT2 cells is very low. BMP2 and 4 are however highly expressed in the murine pituitary and may therefore act as paracrine regulators of gonadotrope function. Relative to equimolar activins, BMPs 2 and 4 only weakly stimulate *Fshb* transcription, but they are nonetheless potent synergistic regulators when applied in combination with the activins. Therefore, physiologically, BMPs may be more important in terms of their cooperative rather than independent actions.

BMP2 and 4 signaling is initiated by the interaction of the ligands with BMP type I receptors, such as BMPR1A and BMPR1B (also known as ALKs 3 and 6). A type II receptor, such as BMPR2, is then recruited into the complex and phosphorylates the type I receptors [374, 435]. The activated type I receptors then phosphorylate intracellular signaling proteins, the most thoroughly characterized of which are the receptor-regulated or R-SMADs, SMAD 1, 5, and 8. Once phosphorylated, R-SMADs form heteromeric complexes with the co-regulatory SMAD (SMAD4), accumulate in the nucleus, and act as transcription factors, either activating or repressing gene expression [250, 374-

375]. Activins stimulate FSH synthesis by up-regulating *Fshb* subunit gene transcription at least in part through the SMAD 2 and 3 signaling proteins [296-297, 300-301]. The available data suggest that BMP2 might preferentially signal through SMAD8 to regulate the *Fshb* gene [282].

BMP family members show some promiscuity in their binding to type I and type II receptors within the TGFβ superfamily. For example, BMP2 and 4 preferentially signal through the type II receptor, BMPR2, but can use ACVR2 in its absence [368]. Similarly, BMPs can bind to several type I receptors, including ACVR1, BMPR1A, and BMPR1B [436]. Each of these type I receptors is expressed in LβT2 cells [282, 292, 405]; however, our previous over-expression data suggested a preferred role for ACVR1 in mediating BMP2 responses [282]. Nonetheless, a role for BMPR1B was not assessed and the data with wild-type and constitutively active BMPR1A yielded conflicting results.

A potential role for BMPR1B in FSH regulation is particularly intriguing in light of the phenotype of so-called Booroola (*FecB*) sheep. These animals show increased ovulation rates, leading to multiple births [412, 437-438], and in some Booroola flocks, FSH levels are elevated [433]. The *FecB* mutation was mapped to the *Bmpr1b* locus and a missense point mutation (CAG→CGG, Q249R) discovered in the highly conserved intracellular serine/threonine kinase domain of the receptor [413, 415-416]; however, the specific alteration in receptor function, at a mechanistic level, has not been determined. Some data suggest that the mutation leads to a partial loss of receptor function, particularly at the ovarian level [414, 439], but alterations at the pituitary level have not been ruled out definitively. In fact, recent data show differences in BMP signaling in pituitary cultures from Booroola and wild-type sheep [418]. These effects may not be mediated directly at the gonadotrope level as previous reports failed to detect BMPR1B expression in ovine gonadotropes by immunofluorescence [408]. Nonetheless, one cannot rule out the possibility of low level expression in these cells that evaded detection by this method. Indeed, *Bmpr1b* mRNA is expressed at low levels in LβT2 cells [282, 292]. Here, we assessed the relative roles of endogenous ACVR1, BMPR1A, and BMPR1B in BMP2-regulated *Fshb* transcription in LβT2 cells and examined potential functional changes in the mutant BMPR1B receptor (Q249R) at the level of the gonadotrope.

Materials and Methods

Reagents

Human recombinant (rh-) activin A and BMP2 were purchased from R&D Systems (Minneapolis, MN, USA). Gentamycin, 1X phosphate buffered saline (PBS), and Dulbecco's Modified Eagle medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate were purchased from Wisent (St-Bruno, Quebec, Canada). 1X Passive Lysis Buffer (PLB) was from Promega (Madison, WI, USA). Protease inhibitor tablets (CompleteMini) were purchased from Roche (Nutley, New Jersey, USA). Aprotinin, leupeptin, pepstatin, phenylmethylsulphonylfluoride (PMSF), SB431542, mouse monoclonal β-actin (#A5441), mouse monoclonal HA (#H9658), and mouse monoclonal MYC (#9E10) antibodies, and rabbit monoclonal FLAG (#F3165) antibody were from Sigma (St. Louis, MO). The pSMAD1/5/8 rabbit polyclonal antibody (# 9511) was from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Bio-Rad (Hercules, California, USA) and enhanced chemiluminescence (ECL) Plus reagent was from GE Healthcare (Piscataway, New Jersey, USA). Compound C (#171261) was purchased from Calbiochem (San Diego, CA). Short-interfering (si) RNAs were purchased from Dharmacon: Control (Cat. # D-001210-05); ACVR1 (Cat. # D-042047-01); BMPR1A (Cat. # D-040598-01); BMPR1B (Cat. # D-051071-01); ACVR2 (Cat. # D-040676-01); ACVR2B (Cat. # D-040629-02); and BMPR2 (Cat. # D-040599-01). Sodium Bisulfite was purchased from Fisher Scientific (Cat.# S654-500) and quinol hydroquinone hydrochinon chinol was purchased from BDH AnalaR (Cat.# 10312).

Constructs

The expression constructs for rat ACVR1-HA, FLAG-ACVR2, FLAG-ACVR2B, and human FLAG-SMAD1 were provided by Dr. Teresa Woodruff (Northwestern University, Evanston, IL, USA). Human BMPR1A-HA (Q233D) and murine BMPR1B-HA (Q203D) were provided by Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan). The following variants were constructed by site-directed mutagenesis using the QuikChange protocol (Stratagene) and the primers in supplementary Table S2.1: constitutively active and siRNA sensitive ACVR1-HA (Q207D); wild-type and siRNA sensitive BMPR1A-Q233D-HA; and wild-type BMPR1B-HA, BMPR1B-Q249R-HA, BMPR1B-Q249R/Q203D-HA, BMPR1B-Q203D/D265A-MYC, methylated BMPR1B-Q249R-HA, and siRNA resistant BMPR1B-Q203D-HA. In the case of methylated BMPR1B-Q249R, primers containing methylated cytosines (Table S2.1) were used and the resulting PCR products purified by ethanol precipitation following *Dpn*I digestion of the parental plasmid and used directly in

transfection experiments. Methylation was confirmed by bisulfite sequencing [440-441]. All BMPR1A and BMPR1B constructs were sub-cloned into pcDNA4 (Invitrogen). This removed the HA tag and replaced it with a C-terminal MYC-HIS tag. Human FLAG-SMAD5 was provided by Dr T. Watanabe (Tokyo University, Tokyo, Japan). The human BMPR2 expression construct [442] and BREX4-luc [443] were provided by Dr. Joan Massague (Memorial Sloan Kettering Cancer Center, New York, NY, USA). The murine *Fshb* promoter-reporter constructs were described previously [296].

Cell cultures and transfections

Immortalized murine gonadotrope LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego, CA, USA) and were cultured in 10% FBS/DMEM and 4 μg/ml gentamycin as described previously [297]. For luciferase assays, cells were plated in 24-well plates (2.5×10^5 cells per well) approximately 36 h prior to transfection. Cells were transfected with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). Twenty-four h after transfection, cells were washed in 1X PBS and then treated with 1 nM (25 ng/ml) activin A and/or BMP2 in DMEM or with DMEM alone (control) for the indicated times. In over-expression experiments, 450 ng of the reporter and 100 ng of each receptor and/or effectors were used per well. Cells were changed into serum-free media 24 h post-transfection. In some experiments, 10 μM of SB431542, an ACVR1B/ACVR1C/TGFBRI inhibitor [349] was included, to block the effects of endogenous activin B (or other ligands signaling through these receptors). In RNA interference (RNAi) experiments, siRNAs were transfected at 5 nM. Resulting data were calibrated to cells transfected with the 1X siRNA buffer only (20 mM KCl, 6 mM HEPES-pH7.5, and 0.2 mM $MgCl_2$) or to those transfected with the control siRNA. Lysates were collected 24 h after transfer to serum-free medium. CHO cells were obtained from Dr. Patricia Morris (Population Council, New York, NY) and cultured in F-12/DMEM containing 10% FBS and 4 μg/ml gentamycin. Except for the BMPR1B/D265A experiment [where 4 μg of FLAG-SMAD1 and 4 μg of receptor were transfected in CHO cells seeded in 10-cm plates], CHO cells in 6-well plates were transfected when 70–80% confluent using Lipofectamine/Plus and 300 ng of the indicated receptor expression vectors and 1 μg of FLAG-SMAD1 or FLAG-SMAD5 for 6 h and then changed to growth media. The repeat of this experiment in LβT2 cells in 6-well plates was performed in a similar way, except Plus reagent and 1200 ng of the indicated receptor expression vectors were included. Cell lysates were then harvested the following day. Human liver carcinoma (HepG2) cells (#HB-8065) were purchased from ATCC and were cultured in 10% FBS/EMEM (modified by ATCC) and 4 μg/ml gentamycin. Transfection protocols were identical to those used for the LβT2 cells.

Luciferase assays

Cells were washed with 1X PBS and lysed in 1X PLB. Luciferase assays were performed on an Orion II microplate luminometer (Berthold detection systems, Oak Ridge, TN, USA) using standard reagents. All treatments were performed in duplicate or triplicate as described in the text or figure legends. Data presented are from at least 2–3 independent experiments.

Immunoblots

Cells were washed with 1X PBS and whole cell protein extracts (WCE) prepared with 1X RIPA (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 0.01 M sodium phosphate pH6.8, 2 mM EDTA, 50 mM sodium fluoride, and CompleteMini Protease Inhibitor Cocktail Tablets) and centrifuged at 13,000 × g for 0.5 h at 4ºC to remove cellular debris. WCEs were subjected to immunoblot analyses as previously described [297]. Briefly, equivalent amounts of protein were separated by SDS-PAGE and transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Blots were probed with the indicated antibodies using standard techniques.

Data Analysis

Data from three replicate experiments were highly similar and their means 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-, two-, or three-way analyses of variance followed by post-hoc pair-wise comparison with Bonferroni or Tukey adjustment where appropriate (Systat 10.2, Richmond, CA, USA), as indicated in the figure legends. Significance was assessed relative to $p < 0.05$.

Results

Transfected BMPR1A and BMPR1B can mediate BMP2 signaling in gonadotropes

Previous transfection studies in our lab suggested that ACVR1 might be the preferred type I receptor mediating BMP2's regulation of *Fshb* transcription [282]. Though we and others [292] observed *Bmpr1b* mRNA expression in both the murine pituitary and LβT2 cells, we did not previously assess its role in BMP2 signaling. In addition, we discovered that the wild-type BMPR1A expression vector we used previously harbored an unwanted frame-shift mutation that truncated the

receptor within the kinase domain. This potentially invalidated the interpretation of our previous results using this reagent. Therefore, we transfected LβT2 cells with the -846/+1 murine *Fshb*-luc reporter and validated wild-type (wt) BMPR1A or BMPR1B receptor expression vectors alone or together with the type II receptor, BMPR2. As observed previously with ACVR1, either BMPR1A or BMPR1B with BMPR2 conferred heightened BMP2-independent and -dependent *Fshb* promoter activity (Figure 2.1A). These effects were only observed when BMPR1A or BMPR1B were expressed in conjunction with BMPR2, but not when either was expressed alone. Similarly, constitutively active forms of BMPR1A (Q233D) and BMPR1B (Q203D) when expressed together with BMPR2, but not alone, stimulated *Fshb* promoter activity (Figure 2.2A). Collectively, these results suggest that over-expressed ACVR1 (as shown previously, [282]), BMPR1A, and BMPR1B can all regulate *Fshb* transcription in conjunction with BMPR2.

BMPR1B harboring the Booroola mutation is fully functional in LβT2 cells

A missense mutation, Q249R, was mapped to the kinase domain of BMPR1B in *Booroola* (FecB) sheep [413, 415-416]. Given that *Bmpr1b* is expressed in the pituitary and may mediate BMP2 effects on *Fshb* (Figs. 2.1A, 2.2A), we investigated the effects of the BMPR1B-Q249R mutation on BMP2 signaling in gonadotrope cells. We introduced the mutation in the context of a murine BMPR1B expression vector. As observed above, expression of type I or type II receptors alone in LβT2 cells had no effect, whereas BMPR1B with BMPR2 up-regulated *Fshb* transcription and this effect was further potentiated in the presence of BMP2 (Figure 2.1B). Unexpectedly, the BMPR1B-Q249R mutant produced equivalent results to the wild-type BMPR1B receptor. Next, we examined potential functional differences between constitutively active forms of BMPR1B and BMPR1B-Q249R. The advantage of this approach is that it allowed us to examine functional changes in BMPR1B-Q249R that were independent of the particular ligand used in our experiments. As seen in Figure 2.2A, BMPR1A-QD, BMPR1B-QD, and BMPR1B-Q249R all stimulated *Fshb* reporter activity when co-transfected with BMPR2 and did so to comparable extents.

To determine whether the results in LβT2 cells were cell-specific, we assessed functionality of the constitutively active BMPR1A and BMPR1B receptors in HepG2 cells. Because *Fshb* reporters are inactive in non-gonadotropes, we used a validated BMP-responsive reporter, BREX4-luc [443]. We previously observed that constitutively active ACVR1 and BMPR1A regulated this reporter in these cells without the need for BMPR2 co-expression (data not shown). Here, BMPR1A-QD, BMPR1B-QD, and BMPR1B-QD-Q249R all stimulated BREX4-luc activity in HepG2 cells and did

so equivalently (Figure 2.2B). Therefore, no obvious functional impairment in BMPR1B-Q249R was noted in two distinct cellular contexts.

BMPR1B-Q249R can stimulate SMAD1/5 phosphorylation

In both LβT2 and HepG2 cells, we failed to detect functional changes in BMPR1B-Q249R. One study used molecular modeling to predict the effects of the Q249R mutation on receptor function and suggested that the mutated receptor might more stably interact with the inhibitory protein, FKBP12 [413]. This would be predicted to impair signaling by the receptor to its downstream effectors, including SMAD 1 and 5 [250, 374-375]. We therefore examined the relative abilities of BMPR1A-QD, BMPR1B-QD, and BMPR1B-QD-Q249R to stimulate SMAD1 and 5 phosphorylation. Use of constitutively active forms of the receptors obviated the need for exogenous ligand treatment. CHO cells were transfected with combinations of the indicated receptors and FLAG-SMAD1 or FLAG-SMAD5. Western blots using a phospho-SMAD1/5/8 antibody showed that all three receptors were equivalent in their abilities to stimulate SMAD1 and SMAD5 phosphorylation (Figure 2.3A, top panel, compare lanes 5, 6, 8, 9, 11, and 12 to lanes 2 and 3). Re-probing of the blots with FLAG (second panel) and MYC (third panel) antibodies confirmed equivalent expression of the SMADs and receptors, respectively. Similar results were observed in LβT2 cells (Figure 2.3B). Thus, the BMPR1B-Q249R receptor appeared capable of stimulating SMAD1/5 phosphorylation to the same extent as the wild-type BMPR1B.

To confirm that point mutations can in fact impair BMPR1B function in these assays, we generated a novel mutation in BMPR1B, D265A. The aspartic acid at position 265 is only 16 amino acids C-terminal to Q249 and is located within the L45 loop of the receptor. This receptor sub-domain has been implicated in SMAD activation by type I receptors [244-246]. The analogous mutation in TGFBR1 (also known as ALK5), D266A, has been reported to impair the ability of the receptor to stimulate SMSAD2 phosphorylation [444]. Whereas BMPR1B-QD and BMPR1B-QD-Q249R stimulated SMAD1 phosphorylation, BMPR1B-QD-D265A was incapable of doing so (Figure 2.3C). All three receptors were expressed at equivalent levels. Thus, our assays are able to detect impairments in receptor function.

BMPR1B-Q249R and wild-type BMPR1B are expressed at similar levels

Given that our analyses failed to show impairments in BMPR1B-Q249R function, we next examined whether the mutation affects receptor expression. Our initial analyses revealed equivalent expression of wild-type and Q249R forms of BMPR1B (Figs. 2.3A-C and data not shown). We noted

that the mutation itself (CAG→CGG) introduces a novel CpG dinucleotide (underlined) that may be a substrate for DNA methylation. Though gene silencing is usually associated with methylation of cytosines in CpGs within promoter or enhancer regions, CpGs within coding regions might also be methylated and therefore have an impact on gene expression through their abilities to bind methyl DNA binding proteins [445]. Although there was no apparent effect of the mutation on expression in transfected cells (Figs. 2.3A-C), the DNA used was propagated in *E. coli* and therefore would not be methylated at this or other CpGs. Therefore, we introduced methylated cytosines on both strands of the BMPR1B-Q249R construct by site-directed mutagenesis using primers methylated specifically at the sites of interest. The same procedure was followed using identical primers that lacked methyl-cytosines. The resulting PCR products were then purified and transfected directly into CHO cells, and their relative expression measured by western blot. Methylation of the amplified DNA was confirmed by bisulfite sequencing (data not shown). Both the methylated and unmethylated BMPR1B-Q249R constructs were expressed to equivalent extents (Figure 2.3D); therefore, methylation at this site alone did not appear to affect receptor expression.

Endogenous BMPR1A mediates BMP2 signaling in gonadotrope cells

Although the data presented above and previously [282] indicated that *Acvr1*, *Bmpr1a*, and *Bmpr1b* are expressed in LβT2 cells and can augment BMP2 actions when over-expressed in this cell line, the data did not definitely show whether BMP2 preferentially signals through one or more of these receptors. First, to confirm that ACVR1, BMPR1A, and/or BMPR1B are required for BMP2 signaling, we treated cells with Compound C (also known as dorsomorphin), a small molecule inhibitor of these three receptors [426]. We treated LβT2 cells with 1 or 10 μM Compound C 30 min prior to treatment with 25 ng/ml BMP2 or activin A for 1 h. At both 1 and 10 μM, the inhibitor significantly impaired BMP2-stimulated SMAD1/5 phosphorylation, but did not affect activin A-stimulated SMAD2 phosphorylation (Figure 2.4A). Increasing the concentration to 20 μM more significantly antagonized the BMP2 effect, but also had a small inhibitory effect on activin A (data not shown). Therefore, in subsequent analyses, we used 10 μM Compound C. We next transfected cells with a murine *Fshb* reporter and treated with BMP2 ± activin A in the presence or absence of Compound C. The inhibitor significantly impaired both BMP2's independent and synergistic actions on *Fshb* transcription, but did not significantly alter the activin A response or basal reporter activity (Figure 2.4B). These data suggested a role for endogenous ACVR1, BMPR1A, and/or BMPR1B in BMP2 signaling in LβT2 cells.

We next knocked down expression of ACVR1, BMPR1A, and/or BMPR1B by RNA interference (RNAi) to determine which might be the preferred receptor in this system. LβT2 cells were transfected with -846/+1 m*Fshb*-luc and siRNAs for ACVR1, BMPR1A, or BMPR1B, and were then treated with 25 ng/ml BMP2 ± 25 ng/ml activin A. We observed the synergistic actions of BMP2 and activin A under control conditions and in the presence of the ACVR1 or BMPR1B siRNAs (Figure 2.5). In contrast, the BMPR1A siRNA significantly inhibited the synergistic actions of BMP2 and activin A on *Fshb* reporter activity, but did not impair the independent activin A response. The BMPR1A siRNA did not significantly diminish the independent BMP2 effect in the context of this analysis, though the trend was in this direction. These data suggested that BMPR1A is the preferred BMP2 type I receptor in LβT2 cells.

We confirmed the functionality and specificity of the siRNAs used in these experiments. LβT2 cells were transfected with epitope-tagged expression vectors for ACVR1, BMPR1A, or BMPR1B that were predicted to be sensitive or resistant to their respective siRNAs based on sequence match or mismatch. That is, we introduced mutations that rendered the expression constructs perfect matches (in rat ACVR1 and human BMPR1A) or created mismatches (in murine BMPR1B) relative to the murine siRNAs used in the experiment in Figure 2.5. In all cases, mutations altered the nucleotide but not amino acid sequences. As shown in supplementary Figure S2.1, the siRNAs specifically impaired expression of their sequence-matched ('sensitive') targets. siRNAs directed against one receptor did not inhibit expression of the other receptors, and sequence-mismatched targets were resistant to their corresponding siRNAs. These data confirmed that the siRNA effects on receptor expression were sequence-specific and did not reflect non-specific or off-target effects.

Although the BMPR1A siRNA specifically impaired murine BMPR1A expression in LβT2 cells, we performed an additional control to show that decreases in *Fshb* reporter activity associated with the BMPR1A siRNA were attributable to receptor knock down and not some other off-target effect. We co-transfected LβT2 cells with -846/+1 murine *Fshb*-luc and combinations of BMPR2 and siRNA-sensitive BMPR1A-QD or siRNA-resistant BMPR1A-QD, along with control, BMPR1A or BMPR1B siRNAs. The two forms of BMPR1A-QD equivalently stimulated reporter activity with BMPR2 (Figure S2.2). The BMPR1A, but not BMPR1B, siRNA inhibited the stimulatory effect of the sensitive, but not resistant, BMPR1A-QD expression vector, confirming that the BMPR1A siRNA effect was sequence-specific.

Endogenous BMPR2 and ACVR2 mediate BMP2 signaling in gonadotrope cells

Finally, having established BMPR1A as the relevant endogenous type I receptor in LβT2 cells, we examined with which endogenous type II it cooperates to mediate BMP2 activity. BMP2 can bind BMPR2, ACVR2, and ACVR2B [369, 397, 446] and we showed previously that all three of these receptors are expressed in LβT2 cells and adult murine pituitary [282]. We co-expressed BMPR1A-QD along with BMPR2, ACVR2, or ACVR2B expression vectors. None of the type II receptors had effects on their own, but all synergized with BMPR1A-QD to stimulate *Fshb* promoter activity (Figure 2.6A). BMPR2 and ACVR2B had more pronounced effects than AVCR2. Next, we knocked down expression of the endogenous type II receptors using siRNAs. Here, we co-transfected cells with the *Fshb* reporter and the indicated siRNAs, and then treated with 25 ng/ml BMP2 in the presence of the activin type I receptor inhibitor, SB431542. Because we showed previously that exogenous BMPs can synergize with endogenous activins in these cells, we needed to remove the potential confounding effects of activins signaling through ACVR2 or ACVR2B. Knockdown of BMPR2 or ACVR2 inhibited both basal activity and the small (though not statistically significant) induction of *Fshb* transcription by BMP2 (Figure 2.6B). The ACVR2B siRNA had no effect.

Discussion

We reported previously that activin A and BMP2 synergistically regulate murine *Fshb* transcription [282]. We postulated that BMP2 might signal preferentially through the type I receptor, ACVR1, to mediate its effects. This was based on the observation that transfection of wild-type ACVR1, but not BMPR1A, with the type II receptor, BMPR2, stimulated promoter-reporter activity alone and in the presence of BMP2. In contrast, constitutively active forms of ACVR1 and BMPR1A both synergized with BMPR2 to stimulate *Fshb* transcription. We subsequently discovered that our presumptive wild-type BMPR1A expression vector possessed a frame-shift mutation, which prematurely truncated the kinase domain of the receptor. Here, when we repeated the analysis using a validated full-length receptor, we observed that BMPR1A functioned similarly to ACVR1 (Figure 2.1A). A third BMP type I receptor, BMPR1B, is also expressed in LβT2 cells [282, 292] and can similarly act in synergy with BMPR2 to regulate *Fshb* transcription. These observations suggest that one or more type I receptor may mediate BMP signaling in gonadotrope cells. Indeed, inhibition of ACVR1, BMPR1A, and BMPR1B with Compound C (dorsomorphin) confirmed a role for at least one of these receptors in BMP2-regulated SMAD1/5 phosphorylation and *Fshb* reporter activity (Figure 2.4)

To more definitely establish which receptor(s) might be most critical, we used siRNAs to deplete endogenous expression of ACVR1, BMPR1A, or BMPR1B. Though all of the siRNAs were effective in depleting expression of their targets in sequence-specific fashion (Figs. S2.1 and S2.2), only BMPR1A knockdown blocked the synergistic actions of BMP2 and activin A on *Fshb* transcription (Figure 2.5). The BMPR1A siRNA did not hinder activin A signaling by itself. These observations suggest that the effect of the BMPR1A siRNA is principally through antagonism of BMP2 signaling. BMP2 can signal through multiple type I and II receptors [363, 368] and there is evidence for functional redundancy of the different receptors. For example, in the absence of BMPR2, BMP2 and 4 can signal through ACVR2 [368]. Here, BMPR2 and ACVR2, but not ACVR2B, appear to mediate the BMP2 response. It was therefore possible that ACVR1 and/or BMPR1B might compensate for the loss of BMPR1A, especially in light of these receptors' ability to modulate *Fshb* transcription in over-expression experiments. However, the almost complete abrogation of BMP2/activin A synergism in the presence of the BMPR1A siRNA (Figure 2.5) and the efficacy of ACVR1 and BMPR1B siRNAs in depleting their targets (Figure S2.1) suggests that neither ACVR1 nor BMPR1B compensates for the loss of BMPR1A in LβT2 cells, at least in these transient transfection assays. In light of these data and those with the type I receptor inhibitor (Figure 2.4), we conclude that BMPR1A is the endogenous signal-propagating BMP2 receptor in these cells. Moreover, because over-expression of BMPR1B can potentiate the BMP2 response, but knock down of the endogenous receptor has no effect, we postulate that BMPR1B may be expressed at insufficient levels to propagate BMP2 signals in these cells.

Some Booroola (*FecB*) sheep, which harbour a missense mutation (Q249R) in BMPR1B, have increased FSH levels [433, 447-448] in association with increased ovulation rates. We therefore hypothesized *a priori* that altered BMPR1B function might contribute to these phenotypes. The data presented here failed to confirm this hypothesis on several levels. First, as described above, though expressed in gonadotrope cells, endogenous BMPR1B does not mediate in BMP2 signaling. Second, the BMPR1B-Q249R receptor was functionally equivalent to wild-type in multiple assays. That is, the wild-type and mutant receptors stimulated two different reporters (*Fshb*-luc and BREx4-luc) in two different cell lines (LβT2 and HepG2) to equivalent extents (Figs. 2.1 and 2.2). Moreover, the receptors similarly stimulated SMAD1 and SMAD5 phosphorylation in CHO and LβT2 cells and were expressed at equivalent levels (Figs. 2.3A-C). Importantly, mutation of a nearby residue, D265A, completely abrogated BMPR1B-regulated SMAD1 phosphorylation (Figure 2.3C), showing the sensitivity of our experimental approach.

We also examined whether the Q249R mutation might affect receptor expression, perhaps through DNA methylation (Figure 2.3D). However, the methylated and unmethylated Q249R receptor were expressed at equivalent levels, which is consistent with a previous report showing equivalent *Bmpr1b* mRNA levels in wild-type and Booroola sheep ovaries [413].

In conclusion, the data presented here show that BMP2 regulates murine *Fshb* subunit transcription both independently and synergistically with activin A by signaling through the type I receptor, BMPR1A, and type II receptors, BMPR2 and ACVR2. Though both ACVR1 and BMPR1B are expressed in LβT2 cells and murine pituitary, and both can act with BMPR2 to regulate *Fshb* promoter activity in over-expression analyses, neither appears necessary for BMP2 action nor does either compensate for the loss of BMPR1A. We further show that the Q249R mutation observed in BMPR1B of Booroola sheep does not alter the ability of the receptor to stimulate SMAD1/5 phosphorylation or activate target gene transcription in different cellular contexts. Future investigations will be required to confirm a role for BMPR1A in FSH regulation in vivo and to determine the nature of altered BMPR1B function in Booroola (*FecB*) sheep.

Acknowledgements

The authors acknowledge Drs. M. Kato, J. Massague, P. Mellon, P. Morris, T. Watanabe, and T. Woodruff for sharing reagents and cell lines. Dr. Paolete Soto and Beata Bak provided valuable feedback on a draft of the ms. Dr. Kathy Lee provided critical preliminary data.

Figure Legends

Figure 2.1: A) LβT2 cells seeded in 24-well plates were transfected with -846/+1 murine *Fshb*-luc and the indicated receptor expression vectors. Cells were then treated in duplicate with 25 ng/ml BMP2 in the presence of 10 μM SB431542 (to remove the effects of endogenous activin B signaling). B) LβT2 cells were transfected and treated as in panel A with indicated receptor expression vectors. Cells were then treated in duplicate with 25 ng/ml BMP2 in the serum-free medium. In both panels, the data presented are the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no receptors or ligands were included. Bars with different symbols were statistically different, whereas those sharing symbols did not differ.

Figure 2.2: A) LβT2 cells seeded in 24-well plates were transfected with -846/+1 murine *Fshb*-luc and the indicated receptor expression vectors. B) HepG2 cells seeded in 24-well plates were transfected with BREX4-luc and the indicated receptor expression vectors. In both panels, cells were starved in serum-free medium for 24 h prior to analysis. The data are the means (+SEM) of three independent experiments and are presented relative to the control group, in which no receptors were transfected. QD, Glu to Asp mutation at position 233 (BMPR1A) or 203 (BMPR1B).

Figure 2.3: A) CHO cells seeded in 6-well plates were transfected with the indicated constitutively active MYC-tagged type I receptors in conjunction with FLAG-tagged SMAD1 or SMAD5. Whole cell protein lysates were subjected to western blot analyses and sequentially probed with pSMAD1/5/8, FLAG, MYC, and β-actin (ACTB) antibodies. B) LβT2 cells seeded in 6-well plates were transfected with the indicated constitutively active type I receptors in conjunction with FLAG-tagged SMAD5. Whole cell lysates were subjected to western blot analyses and sequentially probed with pSMAD1/5/8, FLAG, and ACTB antibodies. C) CHO cells seeded in 10-cm plates were transfected with the indicated constitutively active MYC-tagged type I receptors in conjunction with FLAG-tagged SMAD1. Whole cell lysates were subjected to western blot analysis and sequentially probed with pSMAD1/5/8, FLAG, MYC, and ACTB antibodies. D) CHO cells seeded in 6-well plates were transfected with the indicated amounts of methylated or unmethylated BMPR1B-QD-Q249R DNA constructs obtained directly from site-directed mutagenesis PCR reactions. Whole cell protein lysates were subjected to western blot analyses and sequentially probed with MYC and ACTB antibodies.

Figure 2.4: A) LβT2 cells in 6-well plates were treated with 0, 1, or 10 μM Compound C for 30 min followed by treatment with 25 ng/ml BMP2 (top) or activin A (bottom) for 1 hr. Whole cell lysates were analyzed by western blot for phospho-SMAD1/5/8 or phospho-SMAD2 as indicated. B) LβT2

cells were transfected with the indicated *Fshb* reporter and treated with combinations of activin A and BMP2 in the presence or absence of 10 μM Compound C for 24 h. Data reflect the mean (+SEM) of three independent experiments. Data were log transformed prior to analysis.

Figure 2.5: LβT2 cells seeded in 24-well plates were transfected with -846/+1 murine *Fshb*-luc and 5 nM of the indicated short interfering RNAs (siRNAs) for each of the three BMP type I receptors and treated with 25 ng/ml BMP2 plus/minus 25 ng/ml activin A in serum-free medium. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no siRNAs or ligands were included.

Figure 2.6: A) LβT2 cells seeded in 24-well plates were transfected with -846/+1 murine *Fshb*-luc and the indicated receptor expression vectors. Cells were starved in serum-free medium for 24 h prior to analysis. The data are the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no receptors were transfected. B) LβT2 cells transfected with 846/+1 murine *Fshb*-luc and 5 nM of the indicated short interfering RNAs (siRNAs) for each of the three BMP type II receptors and treated with 25 ng/ml BMP2. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which the control siRNA and no ligands were included.

Supplementary Figure Legends

Figure S2.1: *ACVR1, BMPR1A, and BMPR1B siRNAs specifically inhibit expression of their targeted receptors.* A) CHO cells seeded in 6-well plates were transfected with rat ACVR1-HA (resistant) or a form of the receptor modified to contain the sequence targeted by the murine ACVR1 siRNA (sensitive). Cells were co-transfected with no siRNA or 5 nM control, ACVR1, BMPR1A, or BMPR1B siRNAs. Twenty-four h following transfection, whole cell protein lysates were collected and then subjected to western blot analyses with HA and ACTB antibodies. B) CHO cells were transfected as in panel A with human BMPR1A-myc (resistant) or an BMPR1A-myc construct modified to contain the sequence targeted by the murine BMPR1A siRNA (sensitive). C) CHO cells were transfected as in panel A with murine BMPR1B-myc (sensitive) and a modified form of the receptor containing silent mutations in the BMPR1B siRNA recognition sequence. The blots in B and C were probed sequentially with MYC and ACTB antibodies.

Figure S2.2: *BMPR1A siRNA inhibits BMPR1A-dependent signaling in sequence-specific fashion.* LβT2 cells seeded in 24-well plates were transfected with -846/+1 murine *Fshb*-luc and control DNA (pcDNA3) or BMPR2 in combination with BMPR1A-QD-sensitive or BMPR1A-QD-resistant expression vectors along with no siRNA or control, BMPR1A, or BMPR1B siRNAs. Cells were cultured in serum-free medium for 24 h prior to analysis. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no receptors or siRNAs were transfected.

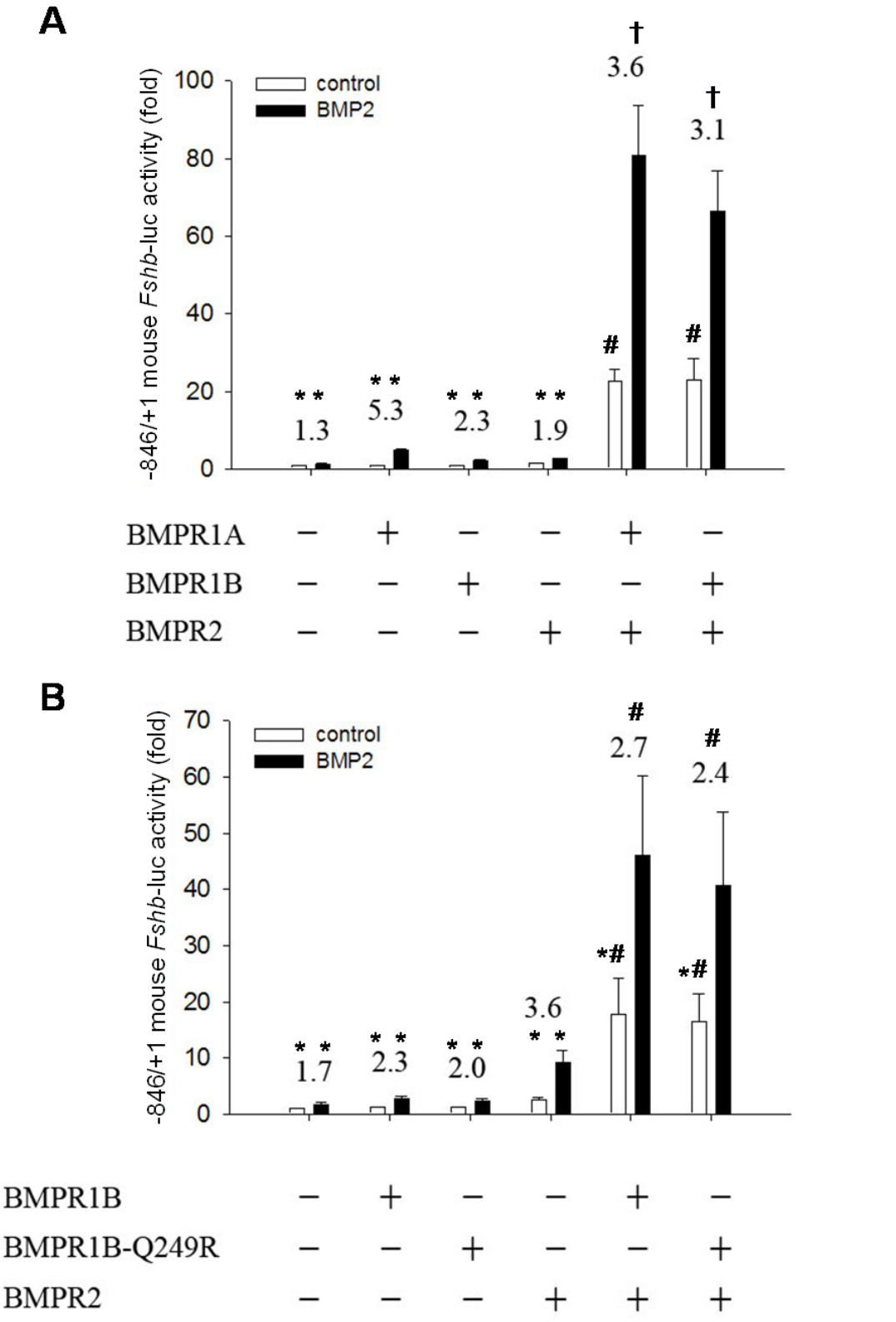
A
-846/+1 mouse Fshb-luc activity (fold)
control
BMP2
100
80
60
40
20
0
1.3
5.3
2.3
1.9
3.6
3.1
BMPR1A − + − − + −
BMPR1B − − + − − +
BMPR2 − − − + + +
B
-846/+1 mouse Fshb-luc activity (fold)
control
BMP2
70
60
50
40
30
20
10
0
1.7
2.3
2.0
3.6
2.7
2.4
BMPR1B − + − − + −
BMPR1B-Q249R − − + − − +
BMPR2 − − − + + +

Figure 2.1

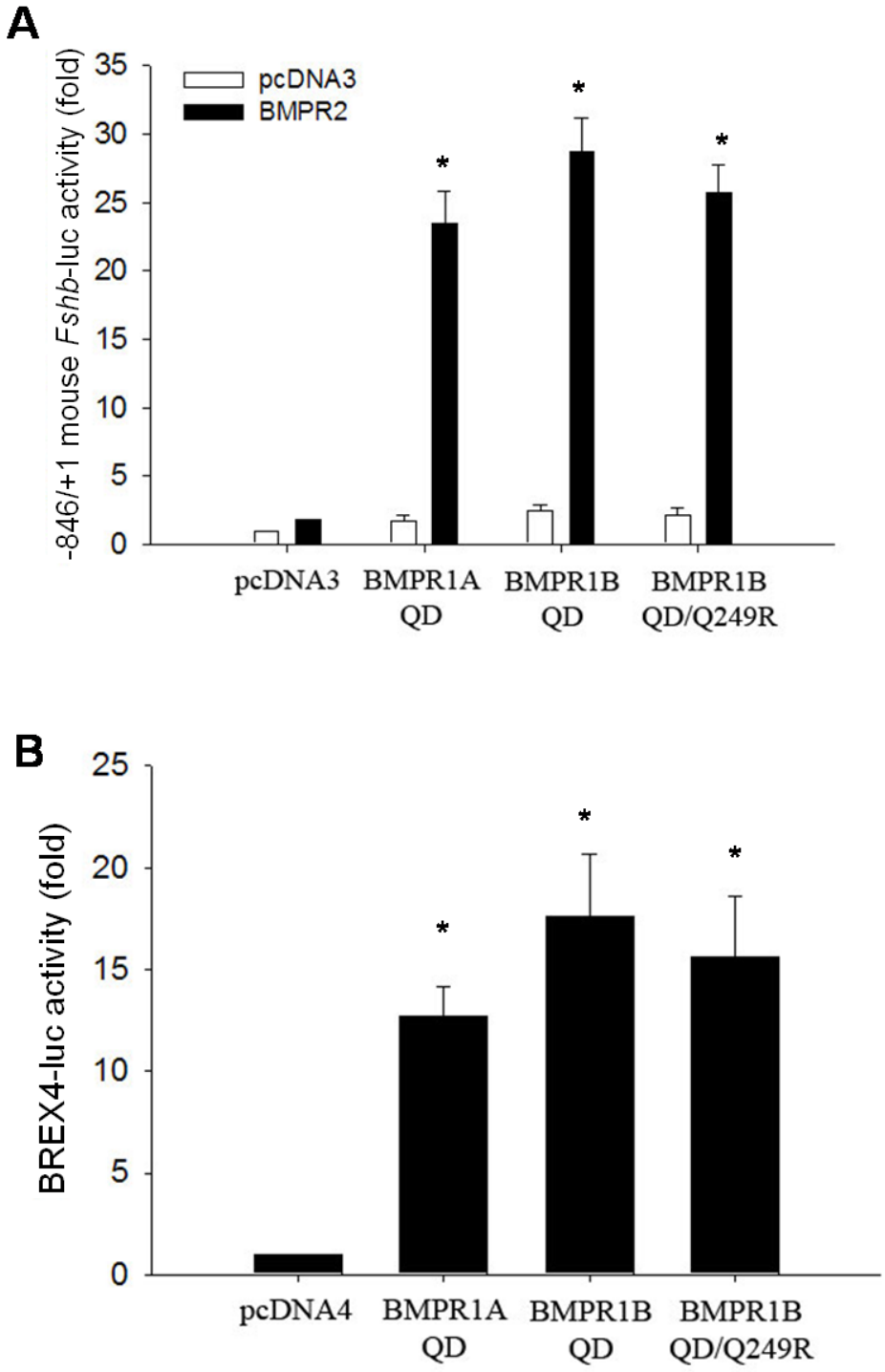
A
-846/+1 mouse Fshb-luc activity (fold)
pcDNA3
BMPR2
0
5
10
15
20
25
30
35
*
*
*
pcDNA3
BMPR1A QD
BMPR1B QD
BMPR1B QD/Q249R
B
BREX4-luc activity (fold)
0
5
10
15
20
25
*
*
*
pcDNA4
BMPR1A QD
BMPR1B QD
BMPR1B QD/Q249R

Figure 2.2

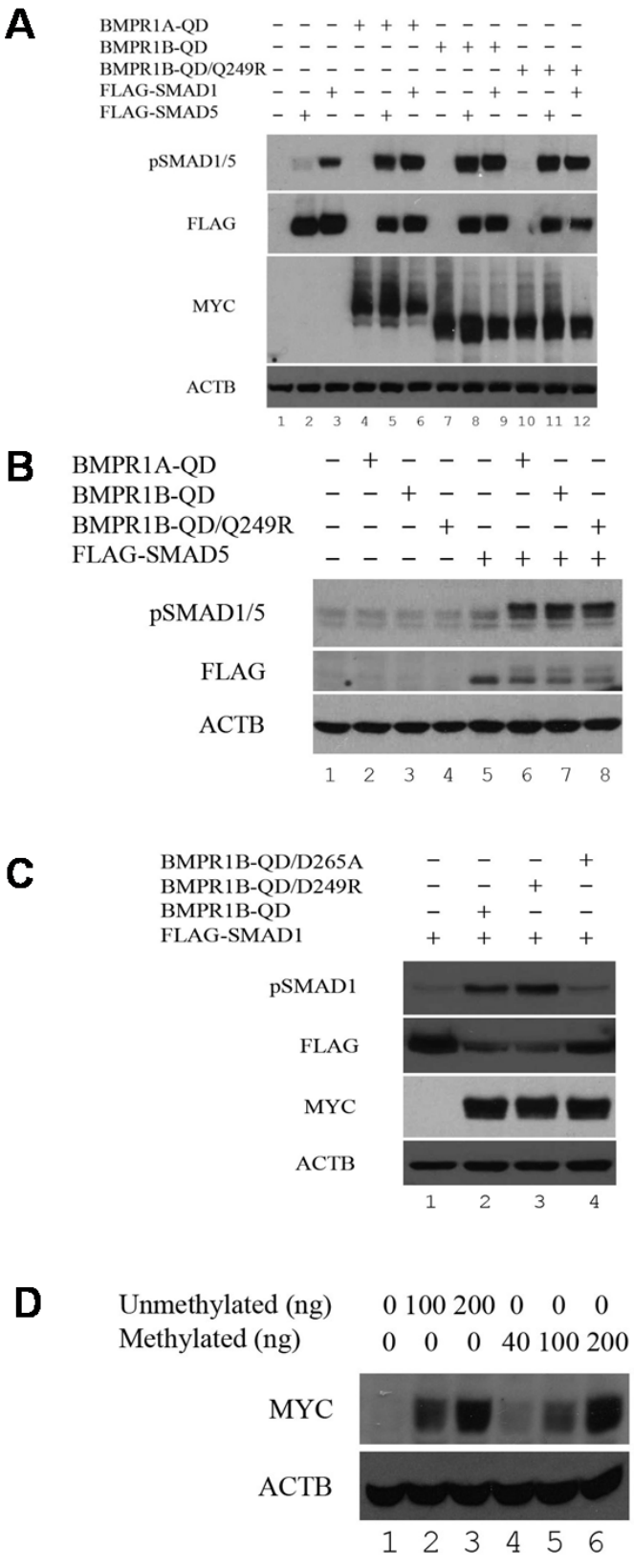
A
BMPR1A-QD
BMPR1B-QD
BMPR1B-QD/Q249R
FLAG-SMAD1
FLAG-SMAD5
pSMAD1/5
FLAG
MYC
ACTB
1 2 3 4 5 6 7 8 9 10 11 12
B
BMPR1A-QD
BMPR1B-QD
BMPR1B-QD/Q249R
FLAG-SMAD5
pSMAD1/5
FLAG
ACTB
1 2 3 4 5 6 7 8
C
BMPR1B-QD/D265A
BMPR1B-QD/D249R
BMPR1B-QD
FLAG-SMAD1
pSMAD1
FLAG
MYC
ACTB
1 2 3 4
D
Unmethylated (ng) 0 100 200 0 0 0
Methylated (ng) 0 0 0 40 100 200
MYC
ACTB
1 2 3 4 5 6

Figure 2.3

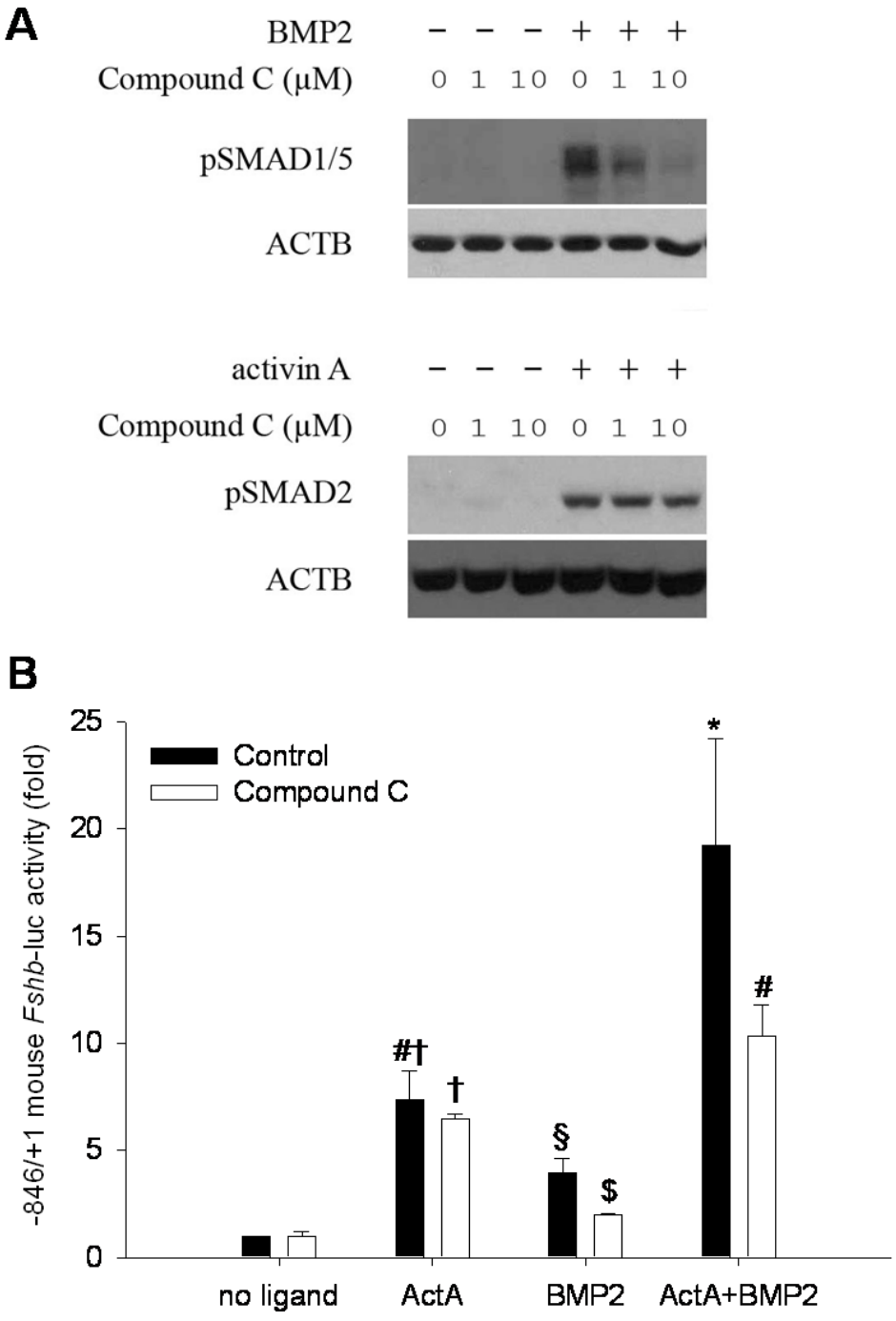
A
BMP2
Compound C (μM)
pSMAD1/5
ACTB
activin A
Compound C (μM)
pSMAD2
ACTB
B
-846/+1 mouse Fshb-luc activity (fold)
Control
Compound C
no ligand
ActA
BMP2
ActA+BMP2

Figure 2.4

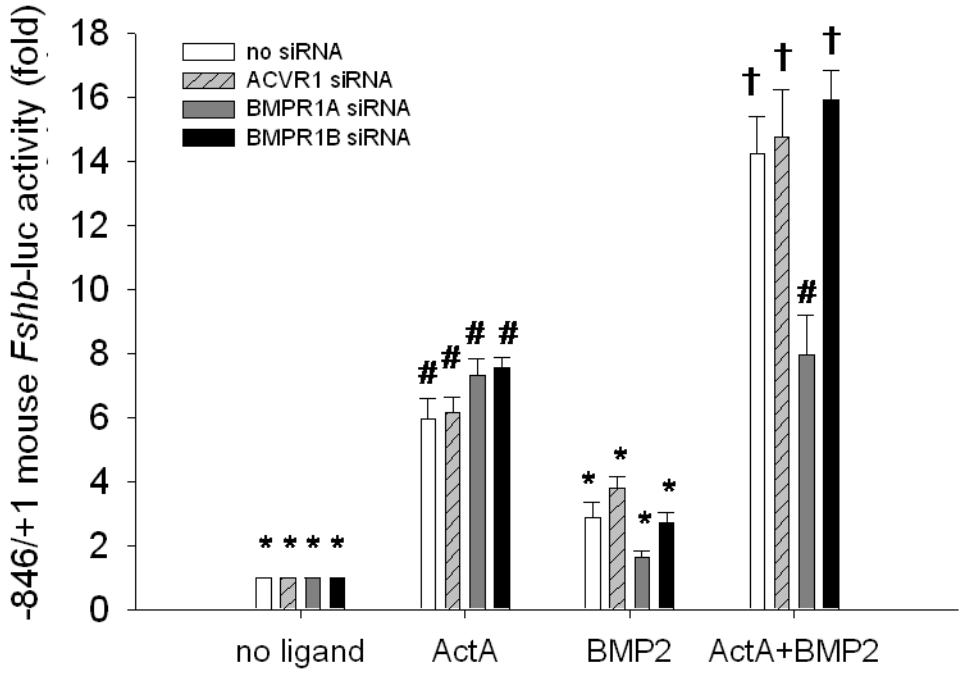
-846/+1 mouse Fshb-luc activity (fold)
18
16
14
12
10
8
6
4
2
0
no siRNA
ACVR1 siRNA
BMPR1A siRNA
BMPR1B siRNA
no ligand
ActA
BMP2
ActA+BMP2

Figure 2.5

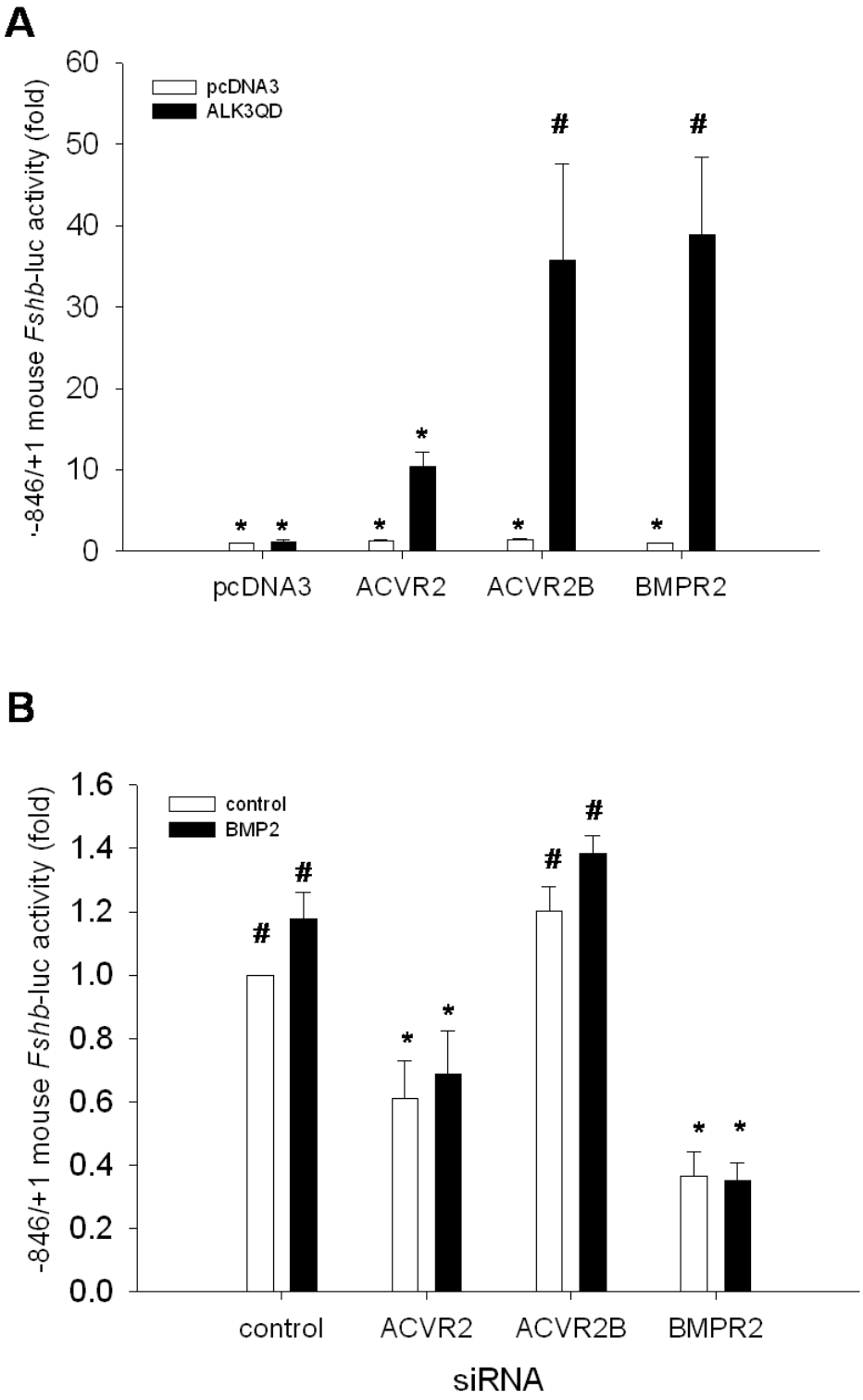
A
-846/+1 mouse Fshb-luc activity (fold)
pcDNA3
ALK3QD
pcDNA3
ACVR2
ACVR2B
BMPR2
B
-846/+1 mouse Fshb-luc activity (fold)
control
BMP2
control
ACVR2
ACVR2B
BMPR2
siRNA

Figure 2.6

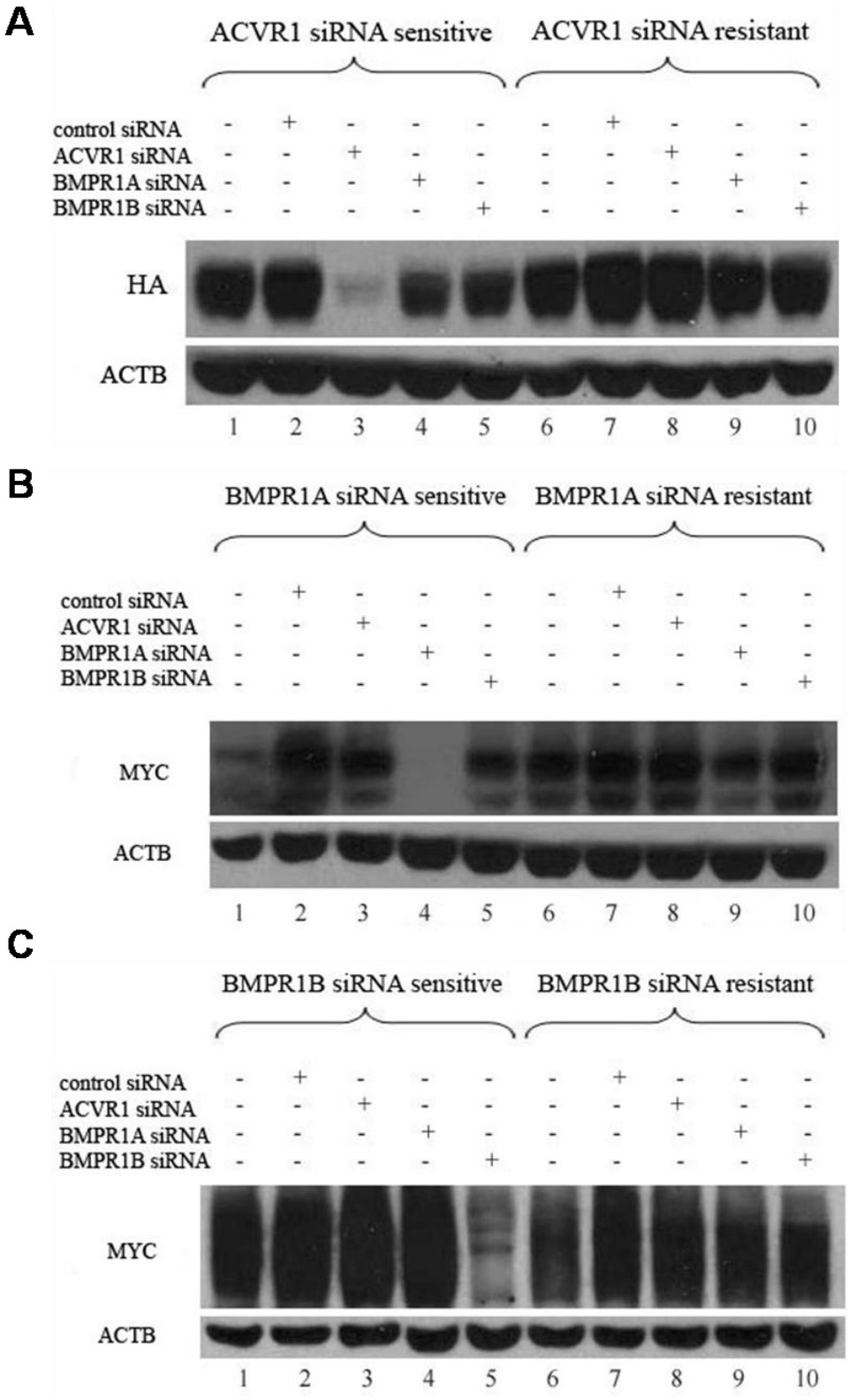
A
ACVR1 siRNA sensitive
ACVR1 siRNA resistant
control siRNA
ACVR1 siRNA
BMPR1A siRNA
BMPR1B siRNA
HA
ACTB
1 2 3 4 5 6 7 8 9 10
B
BMPR1A siRNA sensitive
BMPR1A siRNA resistant
control siRNA
ACVR1 siRNA
BMPR1A siRNA
BMPR1B siRNA
MYC
ACTB
1 2 3 4 5 6 7 8 9 10
C
BMPR1B siRNA sensitive
BMPR1B siRNA resistant
control siRNA
ACVR1 siRNA
BMPR1A siRNA
BMPR1B siRNA
MYC
ACTB
1 2 3 4 5 6 7 8 9 10

Figure S2.1

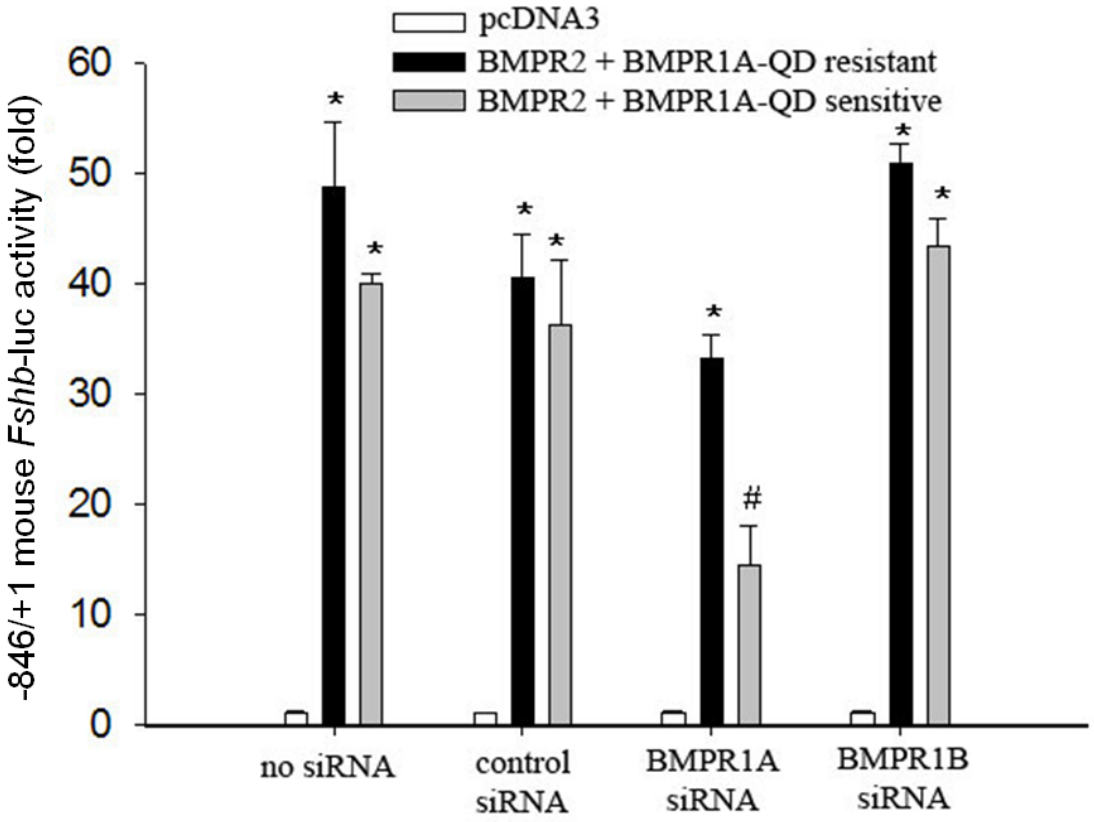
pcDNA3
BMPR2 + BMPR1A-QD resistant
BMPR2 + BMPR1A-QD sensitive
-846/+1 mouse Fshb-luc activity (fold)
60
50
40
30
20
10
0
*
*
*
*
*
*
*
#
no siRNA
control siRNA
BMPR1A siRNA
BMPR1B siRNA

Figure S2.2

Table 2.1. Primers and DNA templates used in site-directed mutagenesis to create their respective plasmids.

Plasmid Name	Forward Primer	Reverse Primer	Template DNA
HA-ALK2 Q207D	AGAGAACTGTGGCTCGAGACATAACCCTGTTGGAGTG	CACTCCACCAGGGTTATGTCTCGAGCCACAGTTCTCT	HA-ALK2
HA- human ALK3	CAGCGAACTATCGCGAAACAAATCCAGATGGTCCGGCAAG	CTTGCCGGACCATCTGGATTTGTTTCGCGATAGTTCGCTG	HA-ALK3 Q233D
HA-mouse ALK6	CCAAAGGACAATAGCTAAGCAGATTCAGATGGTGAAGCAG	CTGCTTCACCATCTGAATCTGCTTAGCTATTGTCCTTTGG	HA-ALK6 QD
siRNA sensitive ALK2QD	CATGCATTCCCAGAGCACAAACCAGCTTGATGTGGGAAAC	GTTTCCCACATCAAGCTGGTTTGTGCTCTGGGAATGCATG	HA-ALK2 Q207D
siRNA sensitive ALK3QD	CGTTGTATCACAGGAGGAATCGTGGAGGAATACCAATTGCCATA	TATGGCAATTGGTATTCCTCCACGATTCCTCCTGTGATACAACG	myc-ALK3 Q233D
siRNA resistant ALK6QD	CTCCAGAAGTGCTGGACGAGTCCCTCAATAGAAACCATTTCCAGTCC	GGACTGGAAATGGTTTCTATTGAGGGACTCGTCCAGCACTTCTGGAG	myc-ALK6 QD
ALK6 Q249R	GAGACTGAGATATATCGGACGGTCCTGATGCG	CGCATCAGGACCGTCCGATATATCTCAGTCTC	HA-ALK6
ALK6QD Q249R	GAGACTGAGATATATCGGACGGTCCTGATGCG	CGCATCAGGACCGTCCGATATATCTCAGTCTC	HA-ALK6QD
ALK6QD D265A	GGGTTCATTGCTGCAGCTATCAAAGGGACTGGG	CCCAGTCCCTTTGATAGCTGCAGCAATGAACCC	myc-ALK6 QD

methyl ALK6QD Q249R	GAGACTGAGATATATmCGGACGGTCCTGATGCG	CGCATCAGGACCGTCmCGATATATCTCAGTCTC	myc-ALK6QD Q249R

m refers to methylated cytosine

Chapter 3

In Chapter 2, I determined the type I and type II receptors used by BMP2 to regulate *Fshb* transcription. Unfortunately, I was unable to determine definitively the relevant R-SMAD(s) in this system because results from siRNA-mediated knock down experiments were inconclusive (data not shown). However, through over-expression assays, Lee et al. suggested that SMAD8 is the R-SMAD BMP2 uses to upregulate *Fshb* transcription. Nonetheless, the mechanisms through which activins and BMP2 synergistically regulate *Fshb* transcription are still unclear. Whereas BMP2 could directly stimulate *Fshb* promoter activity, the majority of data from our lab suggest that that the effect is indirect [282]. LβT2 cells over-expressing SMAD8 were treated with BMP2 at different time points; a steep increase in *Fshb* reporter activity was detected beginning in the third hour, peaking after 8 hours, and remaining stable throughout 24 hours [282]. If SMADs were directly involved in *Fshb* promoter activation, the stimulatory effect should have been detected earlier. This suggests that BMP2 promotes the expression of another protein (or proteins) which then acts to stimulate the *Fshb* promoter. This notion was similarly supported by experiments looking at BMP2 and activin A synergism. BMP2 treatment by itself did not significantly stimulate *Fshb* reporter activity over a 24 h period. In contrast, 24 h activin A treatment stimulated transcription 5-fold. The two ligands applied together produced up to 20-fold stimulation of an *Fshb* promoter-reporter after 24 h. Interestingly, this synergism was only observed beginning 6-8 hours following the onset of BMP2 stimulation, and the elevated reporter activity is maintained up to 24 hours (data not shown in [282]). This intermediate-late response suggests that BMP2 may synergize with activin to regulate *Fshb* expression via an indirect mechanism. Collectively, these data lead us to hypothesize that BMP2 may promote the synthesis of an intermediate protein, possibly a transcription factor, which then works in concert with activins to synergistically promote *Fshb* expression. In Chapter 3, I identified BMP2 induced genes and examined the roles of their protein products in synergistic activation of *Fshb* transcription with activin A.

Title: Bone morphogenetic protein 2 acts via inhibitor of DNA binding proteins to synergistically regulate follicle-stimulating hormone β transcription with activin A

Abbreviated title: BMP2 regulation of FSHβ via Id proteins

Authors: Catherine C. Ho and Daniel J. Bernard

Affiliation: Department of Pharmacology and Therapeutics, McGill University

Corresponding author:

Daniel J. Bernard, Ph.D.

Department of Pharmacology and Therapeutics

McGill University

3655 Promenade Sir William Osler

Montreal, Quebec (Canada)

H3G 1Y6

Ph: 514-398-2525

Fx: 514-398-6705

Email: daniel.bernard@mcgill.ca

Keywords: BMP, FSH, Id1, Id2, Id3, activin A, SMAD3

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Abstract

We recently reported that bone morphogenetic proteins (BMPs) 2 and 4 can stimulate follicle-stimulating hormone β subunit (*Fshb*) transcription alone and in synergy with activins. We further showed that BMP2 signals via the type I receptor BMPR1A (or ALK3) to mediate its effects; however, the intracellular mechanisms through which BMP2 regulates *Fshb* are unknown. In the current study, we used cDNA microarray analyses (and validation by real-time quantitative RT-PCR) to identify BMP2 target genes in the murine gonadotrope cell line, LβT2. siRNA-mediated knockdown, over-expression, and co-immunoprecipitation experiments were used to examine the potential functional roles of selected gene products. Quantitative RT-PCR analysis largely confirmed the results of the array analyses and inhibitors of DNA binding 1, 2, and 3 (Id1, Id2, and Id3) were selected for functional analyses. Knockdown of endogenous *Id2* and *Id3,* but not *Id1,* diminished the synergistic effects of BMP2 and activin A on *Fshb* transcription. Over-expression of Id1, 2, or 3 alone had no effect, but all three potentiated activin A or SMAD3 induction of *Fshb* transcription. Though the precise mechanism through which Ids produce their effects are not yet known, we observed physical interactions between Id1, 2, or 3 and SMAD3. Collectively, the data suggest that BMP2 synergistically regulates *Fshb* transcription with activins, at least in part, through the combined actions of Ids 2 or 3 and SMAD3.

Introduction

The pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play essential roles in reproductive physiology. Perturbations in either the expression or activity of these hormones or their receptors lead to infertility in females and oligospermia or infertility in males [449-452]. Both hormones are secreted from gonadotropes of the anterior pituitary and stimulate gonadal steroidogenesis and gametogenesis. The gonadotropins are heterodimeric glycoproteins (α/β), sharing a common α subunit and unique β subunits. The latter determine both rates of mature hormone synthesis and biological specificity. FSH and LH are regulated by gonadotropin-releasing hormone (GNRH1) secreted from the hypothalamus as well as gonadal sex steroids. FSH synthesis is also regulated by the activins and inhibins, members of the transforming growth factor (TGF) β superfamily [20, 273-274, 319, 453]. Recently, other TGFβ ligands, the bone morphogenetic proteins (BMPs), were shown to stimulate murine *Fshb* transcription alone and in synergy with activins *in vitro* [282, 401, 405, 409]. In contrast, BMP4 was shown to block the stimulatory effect of activins on FSH secretion from sheep pituitary cultures [408], suggesting potential inter-species variation in BMP action.

Several BMP sub-types are expressed in adult murine pituitary and in immortalized gonadotropes, LβT2 [282, 401, 405]; however, the *in vivo* role, if any, for these proteins in FSH regulation have not yet been established. Although BMPs 6 and 7 are endogenously expressed in LβT2 cells, they regulate *Fshb* transcription with low potency. In contrast, BMPs 2 and 4, which are expressed at low levels in these cells, are able to stimulate *Fshb* transcription with greater potency [282]. Because BMPs 2 and 4 are highly expressed in the adult murine pituitary (presumably by other cell types), they may act as paracrine regulators of gonadotrope function.

BMPs 2 and 4 are less potent than activins in their induction of *Fshb* transcription; however, BMPs and activins have strong synergistic actions [282, 401, 405, 409]. Therefore, for *Fshb* regulation, BMPs may be more important for their cooperative than independent actions. In neither case, however, do we have a clear mechanistic understanding of BMP's effects. In Chapter 2, we demonstrated that BMP2 signals via the BMPR1A receptor (also known as ALK3) to stimulate *Fshb* transcription in LβT2 cells [454]. Over-expression approaches also implicated the signaling protein, SMAD8, in BMP2-stimulated *Fshb* expression; but, a role for the endogenous SMAD8 has not yet been established nor do we know how over-expressed SMAD8, directly or indirectly, produces its effects. To gain greater insight into how BMP2 may regulate *Fshb* in gonadotropes, we used cDNA microarrays to identify BMP2 target genes in LβT2 cells. Under our experimental conditions, a

relatively limited number of genes were regulated by BMP2; however, follow-up analyses implicate the inhibitors of DNA binding 2 and 3 (Id2 and Id3) in BMP2/activin A synergistic induction of murine *Fshb* transcription.

Material and Methods

Reagents

Human recombinant (rh-) activin A and BMP2 were purchased from R&D Systems (Minneapolis, MN, USA). Gentamycin, 1X phosphate buffered saline (PBS), and Dulbecco's Modified Eagle medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate were purchased from Wisent (St-Bruno, Quebec, Canada). F12/DMEM with 2.5mM L-glutamine, 15mM HEPES buffer and 1.2g/L sodium bicarbonate was purchased from HyClone Laboratories (South Logan, Utah, USA). Random primers, MMLV-reverse transcriptase, RNasin, and deoxynucleotide triphosphates (dNTPs), and 1X Passive Lysis Buffer (PLB) was from Promega (Madison, WI, USA). Protease inhibitor tablets (CompleteMini) were purchased from Roche (Mississauga, ON, Canada). EZview Red ANTI-FLAG M2 Affinity Gel (Cat. # F2426), SB431542, mouse monoclonal β-actin (#A5441), mouse monoclonal HA (#H9658), and rabbit monoclonal FLAG (#F3165) antibodies were from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA) and enhanced chemiluminescence (ECL) Plus reagent was from GE Healthcare (Piscataway, NJ, USA). Short-interfering (si) RNAs were purchased from Dharmacon (Lafayette, Colorado, USA): Control (Cat. # D-001210-05), IDB1 (ID1, Cat. # D-040701-17); IDB2 (ID2, Cat. # D-060495-02); IDB3 #2 (ID3, Cat. # D-046495-02); IDB3 #3 (ID3, Cat. # D-046495-03). Lipofectamine/Plus, Lipofectamine 2000, TRIzol Reagent, and SYBRgreen Supermix for qPCR were from Invitrogen (Carlsbad, CA, USA). In solution MG132 proteasome inhibitor was purchased from Calbiochem (San Diego, California, USA) (Cat. # 474791).

Constructs

The murine and porcine *Fshb* promoter-reporter constructs were described previously [296, 303]. The SMAD3 responsive $CAGA_{12}$-luc reporter was previously described by Dennler et al. 1998 [455-456]. HA tagged murine Id1, 2, and 3 expression constructs were generously provided by Dr. Nacksung Kim [457]. Human FLAG-SMAD1, human FLAG-SMAD3, and murine FLAG-SMAD4 were provided by Dr. T. Woodruff (Northwestern University, Chicago, IL, USA). Human FLAG-SMAD2 and human FLAG-SMAD3 were provided by Dr. E. Robertson (University of Oxford,

United Kingdom). Murine FLAG-SMAD5 and FLAG-SMAD6 were provided by Dr. T. Watanabe (Tokyo University, Tokyo, Japan). Murine FLAG-SMAD7 and rat myc-SMAD8 were provided by Dr. C.H. Heldin (Ludwig Institute for Cancer Research, Sweden). The rat FLAG-SMAD8 construct was generated in-house by PCR using myc-SMAD8 as template. Human FLAG-SMAD3N, NL, LC, C were purchased from Addgene (Cambridge, MA, USA).

Cell cultures and transfections

Immortalized murine gonadotrope LβT2 cells were provided by Dr. P. Mellon (University of California, San Diego, CA, USA) and were cultured in 10% FBS/DMEM and 4 μg/ml gentamycin as described previously [297]. For gene array experiments and qRT-PCR analyses [see details below], LβT2 cells cultured in 10-cm dishes for approximately 48 h were washed with serum-free DMEM and then treated for 24 h with 2 nM (50 ng/ml) BMP2 in DMEM. The ALK4/5/7 inhibitor SB431542 [349] was included (final concentration 10 μM) to block the effects of endogenous activin B. Cells were washed with 1 x PBS and total RNA extracted with TRIzol (Invitrogen) following the manufacturer's instructions.

For luciferase assays, cells were plated in 24-well plates (2.5 x 10^5 cells per well) or in 48-wells (0.8 x 10^5 cells per well) approximately 36 h prior to transfection. Cells were transfected with 450 ng or 225 ng of the reporter/well, respectively, using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). In Id1, 2, or 3 over-expression experiments, LβT2 cells cultured in 24- or 48-well plates were transfected with 50 or 25 ng of expression plasmid per well, respectively. In both cases cells were treated with 1 nM activin A and lysates collected 24 h after treatment. In SMAD3 and Id1, 2, or 3 over-expression experiments, LβT2 cells cultured in 24- or 48-well plates were transfected with 100ng or 50 ng of the SMAD3 expression plasmid per well. Cells were changed to serum-free media and lysates collected 24 h later. In RNA interference (RNAi) experiments, siRNAs in 1x siRNA buffer (20 mM KCl, 6 mM HEPES-pH7.5, and 0.2 mM $MgCl_2$) were transfected at a final concentration of 5 nM. Resulting data were normalized to cells transfected with the control siRNA. Twenty-four h after transfection, cells were washed in 1X PBS and treated with 1 nM (25 ng/ml) activin A and/or BMP2 in DMEM or with DMEM alone (no ligand control) for 24 h.

CHO cells were obtained from Dr. P. Morris (Population Council, New York, NY, USA) and cultured in F-12/DMEM containing 10% FBS and 4 μg/ml gentamycin. CHO cells in 6-well plates were transfected when 70–80% confluent using Lipofectamine/Plus and 100-250 ng of the indicated Id expression vectors and 10 nM of the indicated control or Id siRNAs for 6 h and then changed to

growth media. Cell protein lysates were harvested the following day for use in western blot analyses [see below]. CHO cells grown in 10-cm dishes were transfected in the same manner as in the 6-well plates using Lipofectamine/Plus and 4 µg of the indicated HA-tagged Id and FLAG-tagged SMAD expression vectors. Cell protein lysates were harvested the following day for use in immunoprecipitation analyses [see below]. In one experiment (as indicated), 10 µM MG132 in growth media was included 5 h prior to harvest.

Gene Array

Affymetrix GeneChips (430 v. 2.0) (Santa Clara, CA, USA) were used to identify BMP2 target genes in LβT2 cells. Total RNA was collected from cells treated as described above and submitted to the Rockefeller University Genomics Resource Center for processing. Two sets of raw chip data (n=2) from the microarrays were analyzed using FlexArray (v. 1.3 from GenomeQuébec) first by background correction then by data normalization. The average difference for each gene between treated and untreated cells was calculated and the fold change in gene expression determined. Supplementary Figure S3.1A shows the mean of two different arrays represented as a scatter plot. Data points that stray furthest from the identity line represent genes that showed the greatest fold change in response to BMP2 treatment. Data were then analyzed using EB Wright & Simon statistical analysis and the results presented as a Volcano Plot (Figure S3.1B). Cluster analysis of the microarray data was performed with the DAVID online functional annotation tool (http://david.abcc.ncifcrf.gov/). The protein products of the regulated genes can be broadly categorized into functional groups implicated in TGFβ signaling, transcriptional regulation, cardiac development, muscle contraction, negative regulation of cellular metabolic process, and one gene encoded a protein of unknown function.

Reverse transcription and quantitative RT-PCR

Reverse transcription was performed on 1-2 µg of total RNA as previously described [297]. qRT-PCR was performed on the resulting cDNA using the SYBRgreen Supermix following manufacturer's instructions with the Corbett Rotorgene 6000 qPCR machine (Corbett Life Science). Data represent the mean of three independent experiments (N=1 per experiment). Expression of target genes was normalized relative to ribosomal protein L19 (*Rpl19*) in the same sample. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method [458-459] and the data presented relative to the no ligand control. Sequences of the qPCR primers for the various target genes are shown in Table S3.1.

Luciferase assays

Cells were washed with 1X PBS and lysed in 1X PLB. Luciferase assays were performed on an Orion II microplate luminometer (Berthold detection systems, Oak Ridge, TN, USA) using standard reagents. All treatments were performed in triplicate as described in the text or figure legends. Data are represented as means of means from three or more independent experiments.

Immunoprecipitation (IP)

Cells were washed with 1X PBS and whole cell protein extracts (WCE) prepared with lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and centrifuged at 10,000 x rpm for 15 min at 4°C to remove insoluble material. WCEs were subjected to immunoprecipitation using EZview Red ANTI-FLAG M2 Affinity Gel following the manufacturer's instructions. In short, WCEs were incubated with the affinity gel on a rotating platform overnight at 4°C to allow binding of FLAG-SMAD3 to the Anti-FLAG affinity gel. The affinity gel was then incubated in 1X FLAG peptide (Cat. # F4799) solution on a rotating platform for 45 min at 4°C to elute gel-bound proteins. The eluted proteins were then analyzed by immunoblot [see below].

Immunoblotting

Cells were washed with 1X PBS and WCEs prepared in 1X RIPA buffer (1% NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH6.8, 2 mM EDTA, 50 mM sodium fluoride, and CompleteMini Protease Inhibitor Cocktail Tablets) and centrifuged at 13,000 × rpm for 0.5 h at 4°C to remove insoluble material. WCEs (or eluted proteins from IPs) were subjected to immunoblot analyses as previously described [297]. Briefly, equivalent amounts of protein were separated by SDS-PAGE and transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Blots were probed with the indicated antibodies using standard techniques.

Data Analysis

Reporter assay and qPCR data from three replicate experiments were highly similar. Therefore, means of treatment replicates within each experiment were calculated to generate a single value per treatment per experiment. The data from replicate (three or more) experiments were then used for statistical analyses. Data are presented as fold-change from the control condition (no ligand and/or transfection with empty vector alone). Differences between means of untransformed or log-transformed data were compared using one-, two-, or three-way analyses of variance (ANOVA)

followed by post-hoc pair-wise comparison with Bonferroni or Tukey adjustment where appropriate (Systat 10.2, Richmond, CA, USA). Significance was assessed relative to $p < 0.05$.

Results

Identification of BMP2-regulated genes by microarray analysis

cDNA microarrays were used to identify BMP2 target genes in LβT2 cells. These cells synthesize activin B [170, 282]. Therefore, to remove effects derived from synergistic actions of exogenous BMP2 and endogenous activin B, we blocked the latter with the small molecule inhibitor, SB431542 [349]. BMP2 (2 nM for 24 h) stimulated an increase in mRNA levels of 18 genes [inhibitor of DNA binding 1 (*Id1*), *Id2, Id3, Asgr1, Atp2c2, Chrna2, Ephx2, Evc2, Gdf9, Gkn3, Hesx1, Klk7, Rgs6, Rya3, Smad6, tbc1d10a, Tnni3,* and *Tnnt1*] (Figure S3.1 and Table 3.1), with the magnitude of change ranging from two to 15 fold. BMP2 also down-regulated seven transcripts [*Calb1, Camk4, Cpa1, Crym, Matn1, Stk25,* and *Tg*] (Figure S3.1 and Table 3.1) by greater than two-fold.

Quantitative real-time PCR (qRT-PCR) was used to validate the results from the microarray analysis. LβT2 cells were treated with BMP2 and SB431542 in the same manner as for the microarray analysis. RNA was collected after 24 h and relative expression levels of nine up-regulated (Figure 3.1A) and three down-regulated genes (Figure 3.1B) were examined by qRT-PCR. These 12 genes were selected based on our interest in their putative functions. The changes in gene expression observed in qRT-PCR mirrored qualitatively those observed with the microarray, though the overall magnitude of the response differed between the two methods. One gene that differed on the array, *Atp2c2,* showed no change in response to BMP2 treatment by qRT-PCR. The correct identity of the PCR products was verified by restriction digest or direct sequencing (data not shown). Although *Fshb* expression in LβT2 cells (BMP2 treated or untreated) was below the threshold of detection on the microarray, we observed a BMP2-stimulated increase by qRT-PCR (data not shown). However, because the *Fshb* mRNA expression level in cells not treated with BMP2 (i.e., control cells) was at or below the detection limit of the qRT-PCR assay, it was difficult to accurately assess the precise fold induction.

Depletion of endogenous *Id2* or *Id3* inhibits both activin A and activin A/BMP2 regulation of *Fshb* transcription

The inhibitors of DNA binding (Ids) 1, 2, and 3 were among the genes most up-regulated by BMP2 (Figs. 3.1A and S3.1, and Table 3.1). Id proteins have previously been implicated as effectors of BMP signaling in a variety of cellular systems [381-388]. To determine a potential role for endogenous Id proteins in BMP2 regulation of *Fshb* transcription, we used siRNAs to deplete their expression in LβT2 cells. Cells were transfected with a murine *Fshb* promoter-reporter and the indicated siRNAs. After 24 h, cells were then treated with 1 nM BMP2 ± 1 nM activin A. Knockdown of *Id2* or *Id3*, but not *Id1*, diminished both activin A and activinA/BMP2 stimulated reporter activity (Figure 3.2). To confirm the efficacy of the siRNAs, we examined the effects of the siRNAs on ID1/2/3 protein expression. Under our assay conditions, transfection efficiency of LβT2 cells is insufficient to obtain an accurate measure of the extent of RNAi-mediated knockdown of mRNA/protein expression on a per cell basis [297] Therefore, to validate the siRNAs, we overexpressed murine HA-ID1/2/3 constructs in CHO cells in the presence or absence of the *Id1/2/3* siRNAs (Figure S3.2). Each siRNA potently inhibited protein expression of its target. Collectively, these data suggest that Id2 and 3 may mediate BMP2's synergistic, but not independent effects on *Fshb* transcription.

Id proteins potentiate the stimulatory effect of activin A on *Fshb* transcription

The data in Figure 3.2 suggested that Id protein expression under both untreated and BMP2-stimulated conditions modulates activin A-induction of *Fshb* transcription. To determine whether Ids can substitute for BMP2 to regulate *Fshb* transcription, we transfected LβT2 cells with a murine *Fshb* promoter-reporter along with Id1, Id2, or Id3 expression constructs. Cells were then treated with 1 nM activin A for 24 h. Id1, 2, or 3 expression alone did not significantly alter *Fshb* reporter activity (Figure 3.3). However, expression of Id1, Id2, or Id3 significantly potentiated the stimulatory effect of activin A. These data suggest that Id regulation of *Fshb* transcription is activin-dependent.

Id proteins functionally interact with SMAD3 to stimulate *Fshb* transcription

Activins stimulate *Fshb* transcription, at least in part, via SMAD3 in LβT2 cells [291, 297, 300]. We therefore examined whether Ids cooperate with SMAD3 to regulate *Fshb*. We transfected LβT2 cells with a murine *Fshb* promoter-reporter along with Id1, Id2, or Id3 expression constructs in the presence or absence of a SMAD3 expression construct. As expected, SMAD3 alone strongly up-regulated murine *Fshb* transcription whereas Id1, 2, or 3 alone did not (Figure 3.4A). However,

transfection of Ids in combination with SMAD3 significantly potentiated the stimulatory effect of SMAD3. The same pattern of results was observed with a porcine *Fshb* promoter-reporter (Figure 3.4B). Though there was a significant main effect of Id over-expression, the SMAD3 x Id interaction was not significant ($p = 0.358$), precluding pair-wise comparisons. In contrast, Ids failed to potentiate SMAD3 induction of the SMAD3/4-responsive reporter $CAGA_{12}$-luc (data not shown). These data suggested that the combined actions of Id proteins and SMAD3 are promoter-specific.

Id proteins physically interact with SMAD3

We next asked whether the functional interaction between Ids and SMAD3 might reflect physical interactions between the proteins. FLAG-SMAD3 and HA-Id1, 2, or 3 were co-transfected in CHO cells. Whole cell lysates were harvested, SMAD3 containing complexes immunoprecipitated (IP) with FLAG affinity gel, and interacting Ids assessed by immunoblot with an HA antibody. All three Id proteins were pulled down with FLAG-SMAD3 (Figure 3.5A), suggesting that the proteins are part of the same complex.

Next, we assessed the SMAD3 sub-domain(s) mediating the interaction with Ids. SMAD3 has three functional domains: the N-terminal MH1 domain (N), a linker domain (L), and the C-terminal MH2 domain (C) [250]. Given that all three Ids physically interacted with SMAD3, we used Id2 as a reference to determine the interacting domain in SMAD3. CHO cells were co-transfected with HA-Id2 and the FLAG-tagged full length SMAD3, SMAD3N, SMAD3NL, SMAD3LC, or SMAD3C expression vectors. Whole cell lysates were collected and subjected to IP/western analysis as in Figure 3.5A. The data show a preferential interaction between Id2 and SMAD3 MH2 domain (Figure 3.5B). The identities of the multiple bands in the SMAD3LC and SMAD3C lanes were not determined; however, the original paper describing these constructs reported the same banding pattern [460].

Id2 selectively interacts with SMAD3 and SMAD8

To determine whether the interaction between Id and SMAD proteins is general or specific, we examined Id2's interaction with all eight mammalian SMAD proteins. CHO cells were co-transfected with HA-Id2 and FLAG-SMAD1, 2, 3, 4, 5, 6, 7, or 8 expression vectors. As in the foregoing analysis, whole cell lysates were subjected to IP/western analysis. Although all SMADs were expressed to roughly equivalent levels, Id2 interacted exclusively with SMADs 3 and 8 (Figure 3.6).

Discussion

We previously demonstrated that BMP2 potentiates activin A-induction of *Fshb* transcription in immortalized murine gonadotropes [282]. In addition, we identified ALK3 (BMPR1A) as the preferred type I receptor mediating BMP2 actions (see Chapter 2). In the current study, we further dissected the downstream pathway(s) through which BMP2 may act to modulate activin A-induction of *Fshb* transcription. We used cDNA microarrays as a search tool for candidate regulators. To our surprise, relatively few genes were BMP2-regulated. This may be attributable to the inclusion of the ALK4/5/7 inhibitor, SB431542, to remove the confounding effects of endogenous activin B. Moreover, we only examined a single time point and dose of BMP2 treatment. Nonetheless, known BMP-response genes *Id1, Id2, Id3,* and *Smad6* were among the up-regulated transcripts [380-388], validating our results. Importantly, the qRT-PCR analysis largely confirmed the results of the array analyses. Because Id proteins have been implicated as effectors of BMP signaling in other contexts [381], we explored their potential roles in *Fshb* transcription.

We suppressed endogenous Id1, 2, and 3 expression with siRNAs. Depletion of Id2 or Id3 attenuated both activin A and activin A plus BMP2-induction of *Fshb* transcription, but did not affect the BMP2 response. In contrast, Id1 knockdown was without effect. These data suggest that BMP2 may synergize with activins, at least in part, via up-regulating Id2 and/or 3 production, but that BMP2's independent effects on *Fshb* transcription do not require stimulated Id expression. Consistent with this hypothesis, Id1, 2, or 3 over-expression potentiated the activin A response, while having no effect on their own. Because Id2 or 3 knockdown also antagonized the independent activin A response, basal levels of Id2 or 3 may play previously unappreciated roles in activin-induced *Fshb* transcription. It is interesting to note that Id1 over-expression, but not knockdown, potentiated the activin A response. We do not currently know the cause of these apparently discrepant results. One possibility is that neither basal nor BMP2-stimulated Id1 protein levels are sufficiently high to modulate activin A's effects. Alternatively, Id2 or Id3 might compensate for the absence of Id1. Data from knockout models are consistent with this latter possibility [381, 461-464].

Activins regulate *Fshb* via SMAD proteins, in particular SMAD3 [291, 297, 300]. Indeed, SMAD3 over-expression is sufficient to stimulate *Fshb* transcription in LβT2 cells. We therefore asked whether Ids modulate SMAD3 activity. SMAD3 potently stimulated murine *Fshb* promoter activity and this effect was potentiated by all three Id proteins, which again had no effect on their own. These data are consistent with our previous observation that BMP2 potentiates the effects of over-expressed SMAD3 [282]. We examined Id modulation of SMAD3 induction of two additional

reporters: porcine *Fshb*-luc and $CAGA_{12}$-luc. As with the murine reporter, the porcine *Fshb* promoter is SMAD3 responsive and synergistically regulated by activin A and BMP2 [282, 303]. The pattern of results with the porcine promoter was highly similar to that with the murine promoter. We observed significant main effects of both SMAD3 and Id over-expression. Unlike the case with the murine promoter, however, the SMAD3 x Id interaction was not statistically significant, suggesting that the combined actions of the proteins were additive rather than synergistic. This could reflect inter-species differences or perhaps limitations of the analyses. We prefer the latter explanation. That is, with additional replications of the experiment (and more statistical power), the interaction might have been significant. Indeed, one should note that the Ids when expressed alone did not modify reporter activity. Instead, their actions were only observed in the presence of co-expressed SMAD3. In contrast, Ids failed to potentiate or modify SMAD3 induction of the SMAD3/4-responsive reporter, $CAGA_{12}$-luc (data not shown) [455-456]. The data therefore suggest that SMAD3/Id cooperativity (either synergism or additivity) reflects a promoter-specific rather than a general phenomenon (i.e., that Ids to not generally modify SMAD3 activity).

We next asked whether a physical interaction might underlie the cooperative actions of Ids and SMAD3. By co-immunoprecipitation, we observed interactions between all three Ids examined and SMAD3. We further mapped the interaction to the SMAD3 MH2 domain. To our knowledge, this is the first demonstration of SMAD3-Id interactions. Ids are members of the helix-loop-helix (HLH) family of transcription factors. Interestingly, SMAD3 was similarly shown to interact with MyoD, a basic helix-loop-helix (bHLH) transcription factor, via its MH2 domain [465]. Thus, though we did not map the interaction domain in the Ids, the observation that all three Ids and MyoD interact with SMAD3, suggests that the common HLH domain likely mediates the interaction. Given the conservation of the MH2 domain across the eight SMAD family members in mammals, we were surprised to see that SMAD8 was the only other SMAD to interact with Id2. The basis for this specificity is not yet known. However, it may be important to note that we previously observed that over-expressed SMAD8, but not other BMP-regulated SMADs (1 and 5), potently stimulates murine *Fshb* promoter activity in BMP2-treated LβT2 cells [282].

Though SMAD3 and Id proteins can physically interact, how this might lead to their functional interaction is not yet known. We examined whether co-expression of Ids impacted SMAD3 nuclear localization, but observed no effect (data not shown). SMAD binding to DNA, which is weak on its own, is enhanced through protein-protein interaction. That is, SMADs can interact with high(er) affinity DNA binding co-factors [250, 443, 466-473]. It is therefore tempting to speculate that Ids might increase SMAD3 affinity for the *Fshb* promoter; however, there is no evidence the Ids

bind directly to DNA. Another possibility is that a bHLH family member might interact with SMAD3, inhibiting its activity at the *Fshb* promoter [474]. Ids might then compete for binding to SMAD3, relieving this repression. Future investigations aimed at identifying bHLH proteins interacting with SMAD3 in LβT2 cells will provide an important first step in testing this hypothesis.

In summary, we used global gene expression profiling to identify BMP2-regulated genes in LβT2 cells. Among the up-regulated transcripts were the inhibitors of DNA binding (Id), which are known BMP-response genes in other cellular systems [380-388]. By RNAi-mediated knockdown, we demonstrate that Id2 and Id3 contribute to activin A and activinA/BMP2 regulation of *Fshb* expression. How the Ids produce their effects are not entirely clear. However, over-expression and co-immunoprecipitation data suggest that a physical interaction between Ids and SMAD3 may form part of the underlying mechanism.

Acknowledgments

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Figure Legends

Figure 3.1. LβT2 cells seeded in 10-cm dishes were treated with vehicle or 50 ng/ml BMP2 in SF-DMEM containing 10 μM SB431542 for 24h. RNA was extracted and changes in expression of the indicated genes measured by qRT-PCR. Data (the mean+SEM of three independent experiments) are normalized to the housekeeping gene, *Rpl19*, and presented as fold change in mRNA expression relative to untreated cells. Bars with asterisks were statistically different from 1, Bonferroni adjustment ($p < 0.05$). Up- and down-regulated genes are shown in panels A and B, respectively.

Figure 3.2. LβT2 cells seeded in 24-well plates were transfected with murine -846/+1 *Fshb*-luc and 5 nM of the control short interfering RNA (siRNA) or siRNAs for the indicated *Id*s and treated with 25 ng/ml activin A, BMP2, or both for 24 h in serum-free medium. The data reflect the mean (+SEM) luciferase activity from three independent experiments and are presented relative to the control group, in which control siRNA was transfected but no ligands were included. Bars with asterisks are statistically different from their respective control with the same treatment conditions, Bonferroni adjustment ($p < 0.05$).

Figure 3.3. LβT2 cells seeded in 24- or 48-well plates were transfected with -846/+1 *Fshb*-luc along with 50 or 25 ng/well respectively, of the indicated Id1, Id2, or Id3 expression constructs. Cells were then treated with 25 ng/ml activin A for 24 h. Data are the means (+SEM) of five independent experiments and are presented relative to untreated cells transfected with the empty expression vector. Bars with different symbols were statistically different, whereas those sharing symbols did not differ, Bonferroni adjustment ($p < 0.05$).

Figure 3.4. A) LβT2 cells seeded in 24- or 48-well plates were transfected with -846/+1 *Fshb*-luc along with 50 or 25 ng/well respectively, of the indicated Id1, Id2, and/or Id3 expression constructs and 100 or 50 ng/well respectively, of the SMAD3 expression construct. Cells were cultured in serum-free media 24 h before harvest. Data are the means (+SEM) of eleven independent experiments and are presented relative to cells transfected with the empty expression vectors. Statistical analysis was done with log-transformed data with Tukey adjustment. Bars with different symbols were statistically different, whereas those sharing symbols did not differ ($p < 0.05$). B) LβT2 cells seeded in 24-well plates were transfected with a porcine -326/+8 *Fshb-luc* reporter construct along with 50ng/well of the indicated Id1, Id2, and/or Id3 expression constructs and 100 ng/well of the SMAD3 expression construct. Cells were cultured in serum-free media 24 h before harvest. Data are the means (+SEM) of three independent experiments and are presented relative to cells transfected with the empty expression vectors.

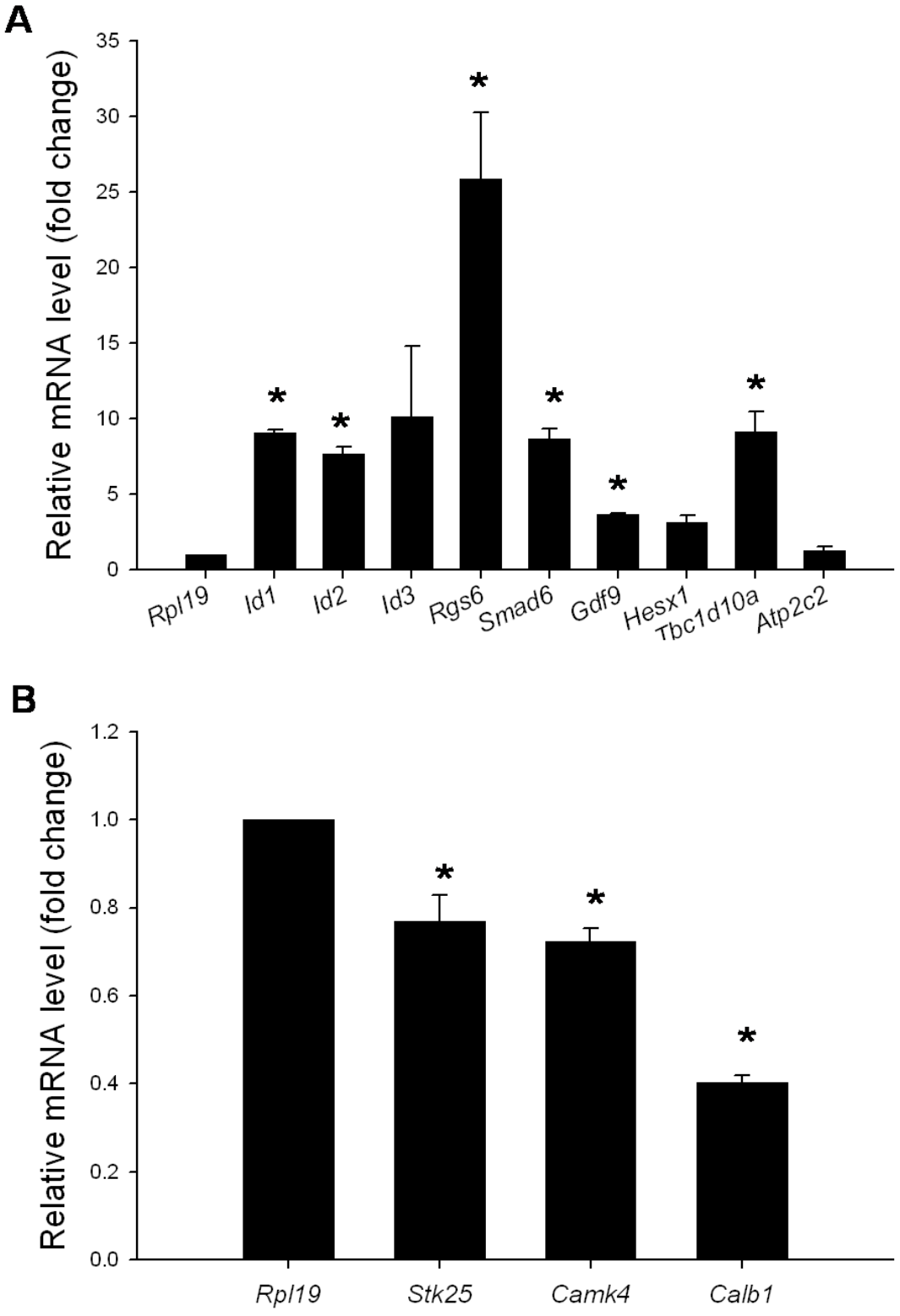
Figure 3.5. A) CHO cells seeded in 10-cm dishes were transfected with 4 µg of HA-Id1, 2, or 3 and 4 µg of FLAG-SMAD3 expression vector. Whole cell lysates were collected for FLAG-immunoprecipitation (IP) analysis. Immunoprecipitated proteins were subjected to immunoblot (IB) analysis with FLAG and HA antibodies. B) CHO cells seeded in 10-cm dishes were transfected with 4 µg of HA-Id2 and 4 µg of FLAG-SMAD3 (WT), FLAG-SMAD3N (N), FLAG-SMAD3NL (NL), FLAG-SMAD3LC (LC), or FLAG-SMAD3C (C) expression vectors. Cells were treated with 10 µM MG132 5 h prior to harvest. IP westerns were performed as in panel B.

Figure 3.6. CHO cells seeded in 10-cm dishes were transfected with 4 µg of HA-Id2 and 4 µg of FLAG-SMAD1, 2, 3, 4, 5, 6, 7 or 8 expression vectors. IP westerns were performed as in Figure 3.5.

Supplementary Figure Legends

Figure S3.1: LβT2 cells seeded in 10-cm plates were treated with 50 ng/ml BMP2 for 24 h in DMEM (containing 10 μM SB431542) before RNA was harvested. Using Affymetrix GeneChips (430 v. 2.0) changes in the expression of 30,000 probe sets were analyzed. A) The mean of two experiments represented as a scatter plot (n=2). B) Data were analyzed using EB statistical analysis software (Wright & Simon) and the results presented as a volcano plot. All the points above 4.32 on the y-axis represent genes that showed a change in expression with a *p*-value of 0.05 or less. All the points beyond +1 on the x-axis represent genes that showed a two-fold or greater increase in expression, and the points before -1 on the x-axis represent genes that showed a two-fold or more decrease in expression. Highlighted points represent genes later verified by qRT-PCR.

Figure S3.2: CHO cells seeded in 6-well plates were transfected with 100-250 ng/well of murine HA-Id1, HA-Id2 or HA-Id3 expression construct. Cells were co-transfected with no siRNA or 10 nM control, *Id1*, *Id2*, or *Id3* siRNAs. Twenty-four h following transfection, whole cell protein lysates were collected and then subjected to immunoblot analyses with HA and β-actin (ACTB) antibodies.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25
20
15
10
5
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*
*
*
*
*
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Id2
Id3
Rgs6
Smad6
Gdf9
Hesx1
Tbc1d10a
Atp2c2
B
Relative mRNA level (fold change)
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1.0
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Rpl19
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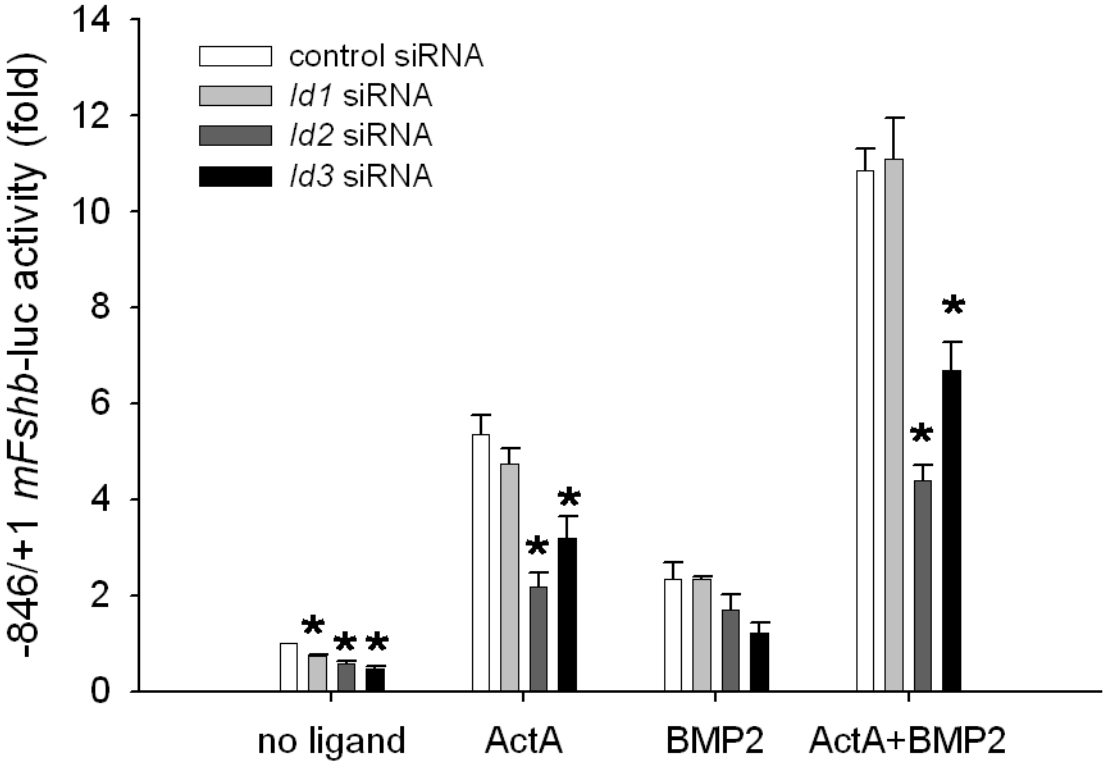
Figure 3.1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8
6
4
2
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control siRNA
Id1 siRNA
Id2 siRNA
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*
*
*
*
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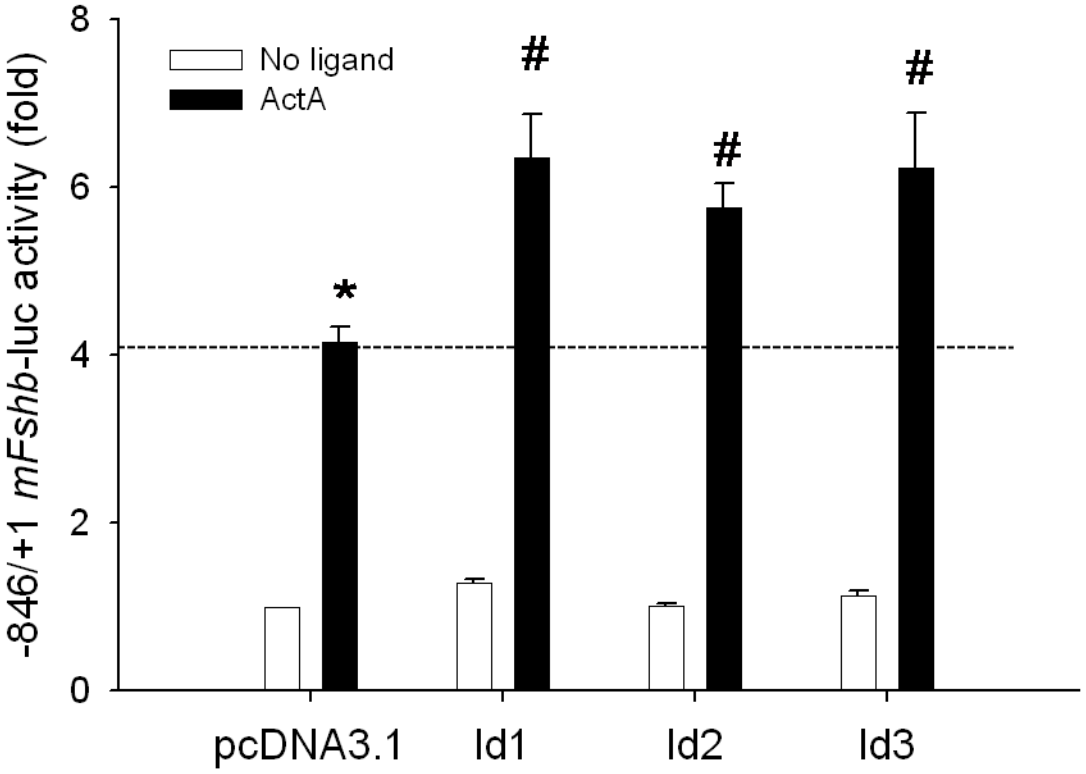
no ligand
ActA
BMP2
ActA+BMP2

Figure 3.2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ActA
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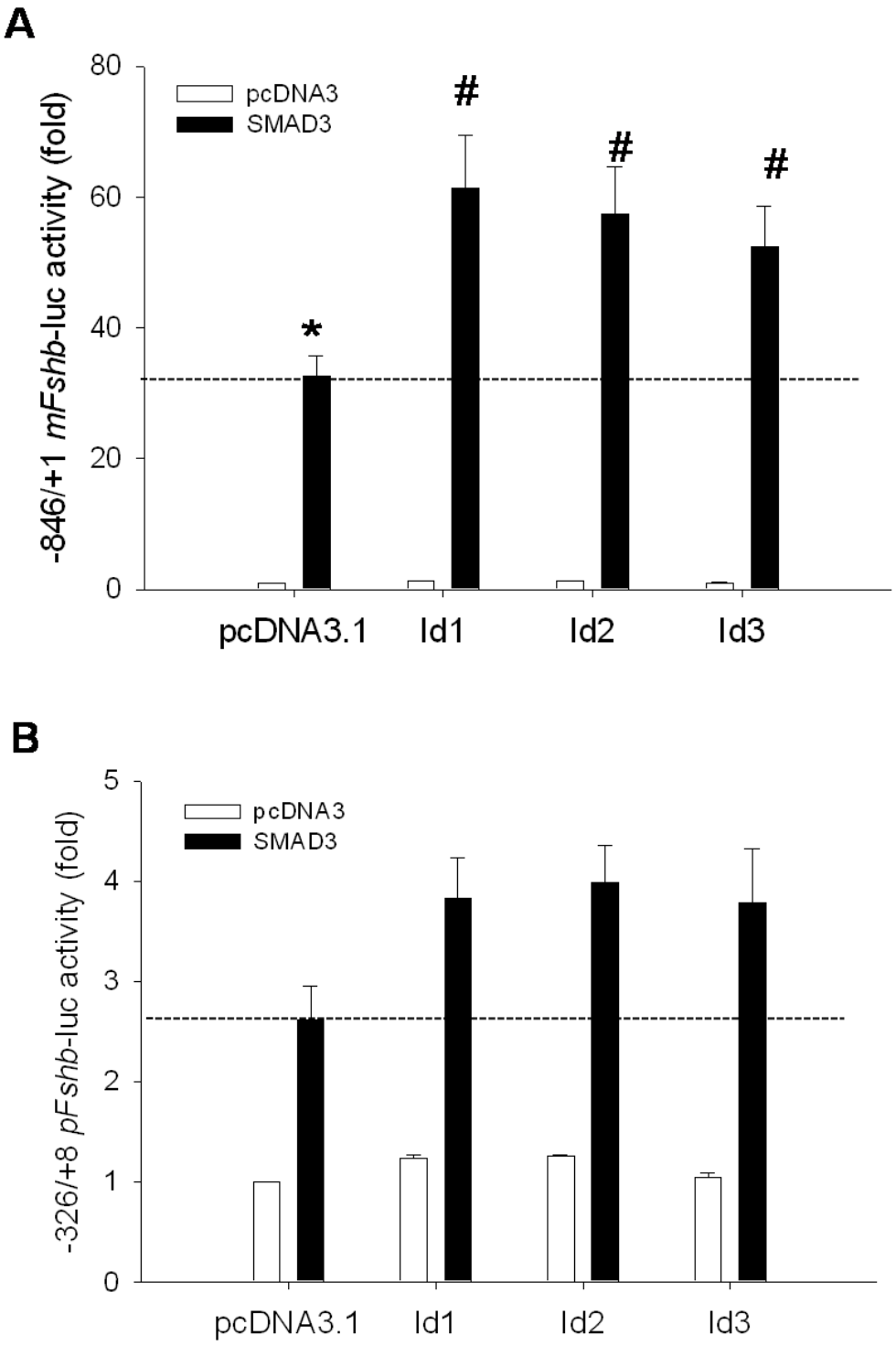
Id1
Id2
Id3

Figure 3.3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40
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SMAD3
-846/+1 mFshb-luc activity (fold)
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Id1
Id2
Id3
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5
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SMAD3
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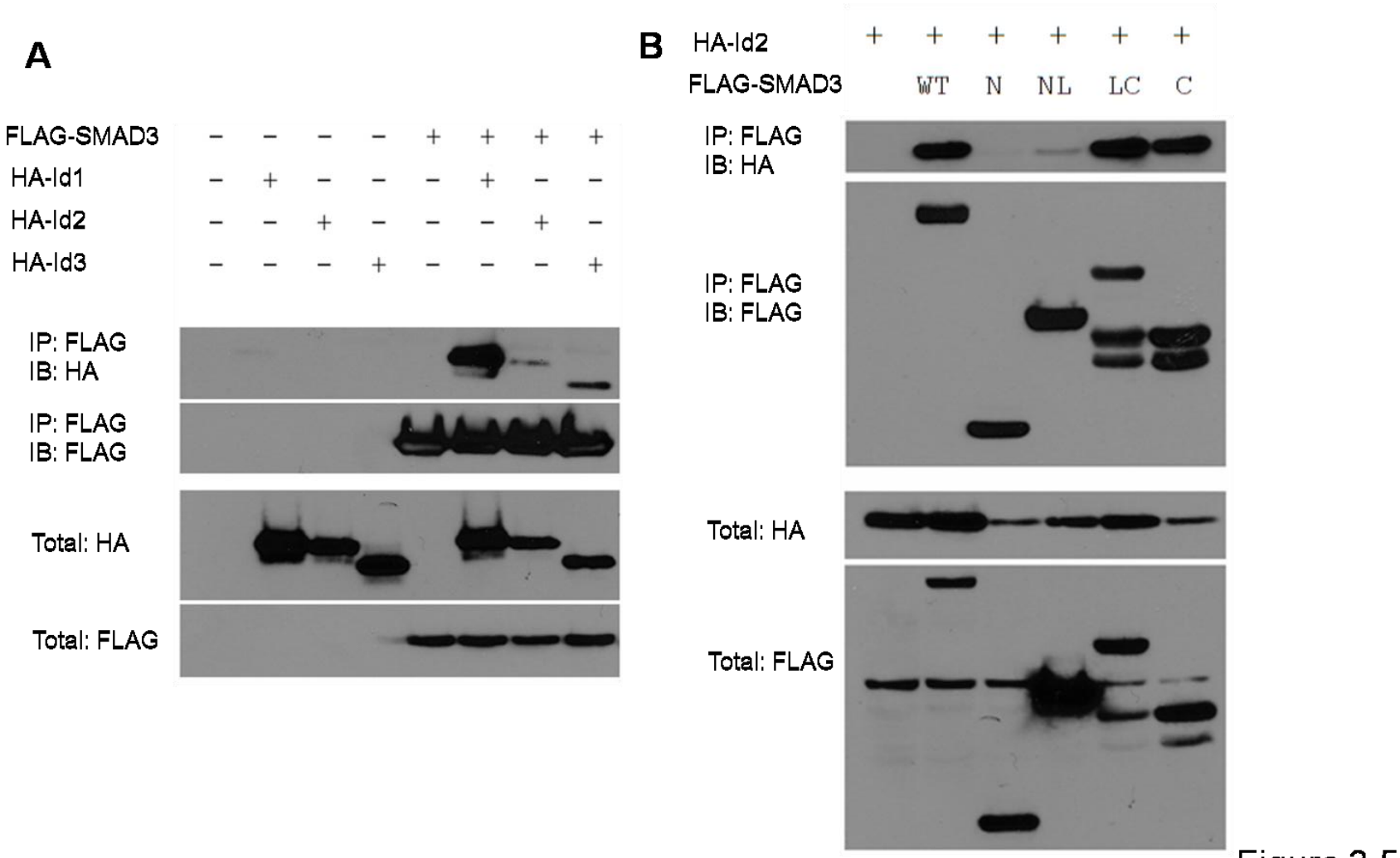
pcDNA3.1
Id1
Id2
Id3

Figure 3.4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HA-Id2 − − + − − − + −
HA-Id3 − − − + − − − +
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IB: HA
IP: FLAG
IB: FLAG
Total: HA
Total: FLAG
B
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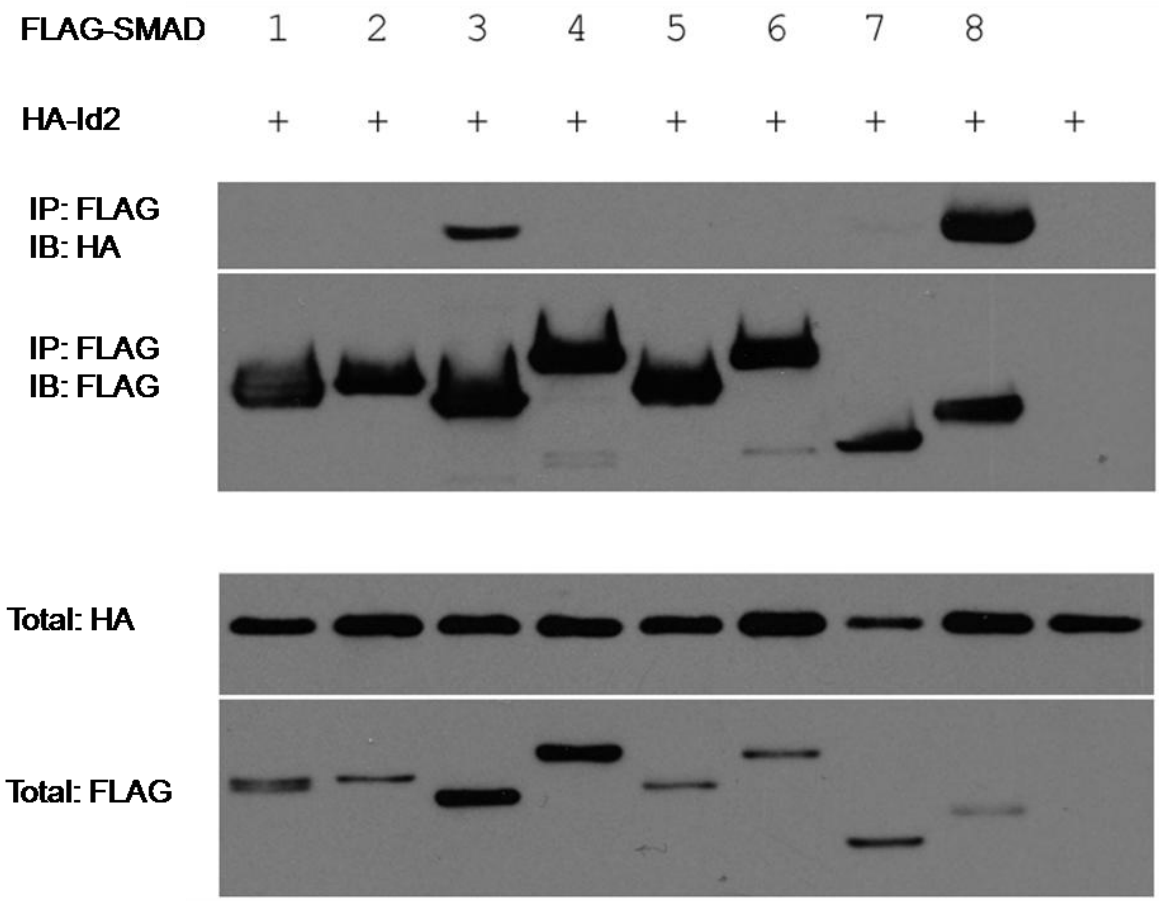
Figure 3.5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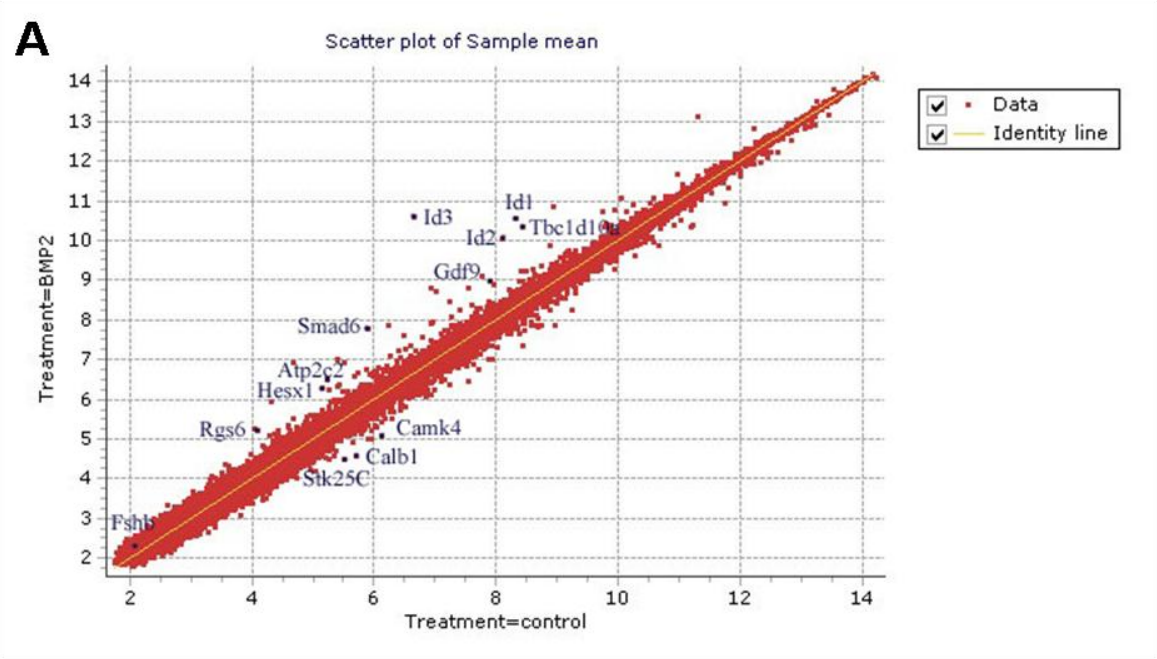
IB: HA
IP: FLAG
IB: FLAG
Total: HA
Total: FLAG

Figure 3.6


A
Scatter plot of Sample mean
Treatment=BMP2
Treatment=control
Data
Identity line
Id3
Id1
Id2
Tbc1d10a
Gdf9
Smad6
Atp2c2
Hesx1
Rgs6
Camk4
Calb1
Stk25C
Fshb

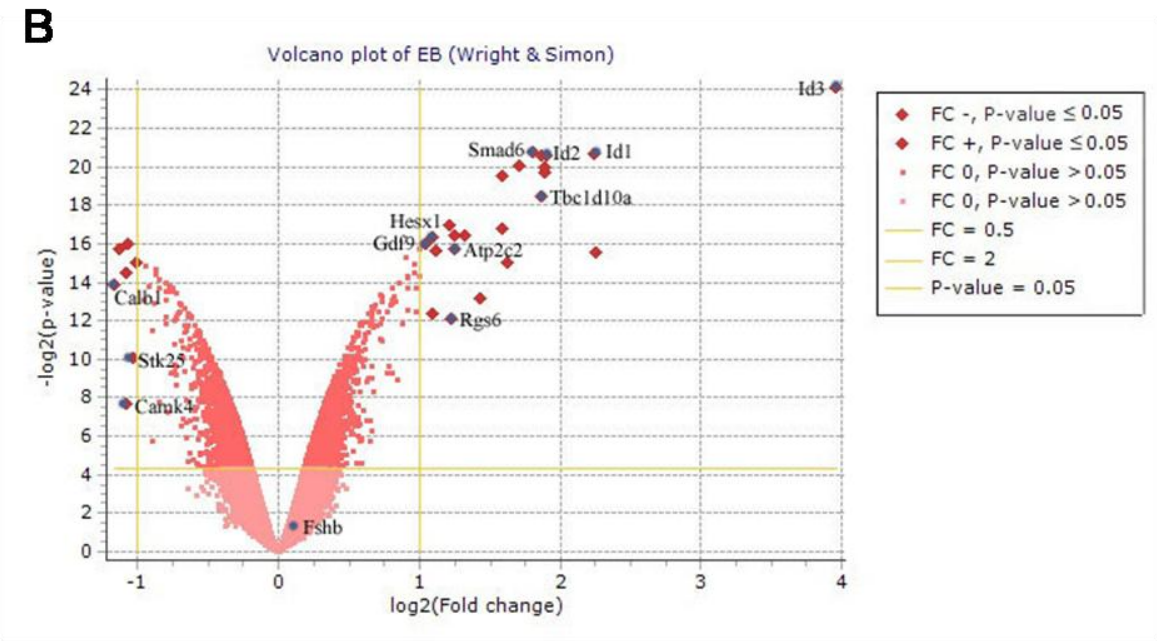

B
Volcano plot of EB (Wright & Simon)
-log2(p-value)
log2(Fold change)
FC -, P-value ≤ 0.05
FC +, P-value ≤ 0.05
FC 0, P-value > 0.05
FC 0, P-value > 0.05
FC = 0.5
FC = 2
P-value = 0.05
Id3
Smad6
Id2
Id1
Tbc1d10a
Hesx1
Gdf9
Atp2c2
Calb1
Rgs6
Stk25
Camk4
Fshb

Figure S3.1

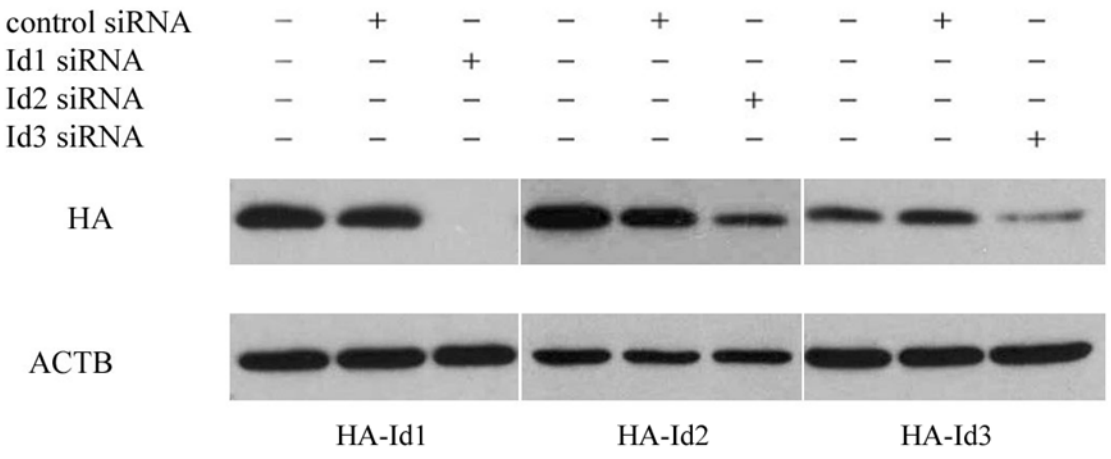

control siRNA
Id1 siRNA
Id2 siRNA
Id3 siRNA
HA
ACTB
HA-Id1
HA-Id2
HA-Id3

Figure S3.2

Table 3.1: BMP2-regulated genes in LβT2 cells.

Gene	**qRT-PCR (fold change)**	**Microarray (fold change)**	**p-value (microarray)**
Inhibitor of DNA binding 3 (Id3)	10.1	15.6	5.5E-08
*Gastrokine 3 (Gkn3) **	-	4.8	2.1E-05
Inhibitor of DNA binding 1 (Id1)	9.1	4.7	5.8E-07
Inhibitor of DNA binding 2 (Id2)	7.7	3.8	6.5E-07
*Troponin I, Cardiac (Tnni3) **	-	3.7	1.0E-06
TBC1 domain family, member 10A (Tbc1d10a)	9.2	3.7	2.7E-06
MAD homolog 6 (Smad6)	8.7	3.6	6.3E-07
*Antimicrobial peptide RYA3 (Rya3) **	-	3.5	5.5E-07
*Ellis van creveld syndrome 2 homolog (Evc2) **	-	3.3	9.2E-07
*Cholinergic receptor, nicotinic, alpha polypeptide 2 (Chrna2) **	-	3.0	1.4E-06
*Troponin T1, skeletal, slow (Tnnt1) **	-	2.4	1.1E-05
ATPase, C++ transporting, type2C, member 2 (Atp2c2)	1.2	2.4	1.8E-05
Regulator of G-protein signaling 6 (Rgs6)	25.9	2.3	2.3E-04
*Epoxide Hydrolase 2 (Ephx2) **	-	2.3	7.6E-06

*Kallikrein 7 (Klk7) **	-	2.2	2.0E-05
Homeo box gene expressed in ES cells (Hesx1)	3.1	2.1	1.2E-05
*Asialoglycoprotein receptor 1 (Asgr1) **	-	2.1	1.8E-04
Growth Differentiation factor 9 (Gdf9)	3.7	2.1	1.6E-05
*Thyroglobulin (Tg) **	-	0.5	2.9E-05
Serine/Threonine kinase 25 (Stk25)	0.8	0.5	9.0E-04
*Matrilin 1 cartilage matrix protein 1 (Matn1) **	-	0.5	1.5E-05
Calcium/calmodulin-dependent protein kinase IV (Camk4)	0.7	0.5	4.8E-03
*Crystallin, Mu (Crym) **	-	0.5	4.3E-05
*Carboxypeptidase a1 (Cpa1) **	-	0.5	1.8E-05
Calbindin-28K (Calb1)	0.4	0.4	6.7E-05

*Indicates genes not analyzed by qRT-PCR

Supplementary Table S3.1: Sequences of primers used in qRT-PCR analyses

Gene	Forward	Reverse
Rpl19	5'CGGGAATCCAAGAAGATTGA3'	5'TTCAGCTTGTGGATGTGCTC3'
Id1	5'GGTACTTGGTCTGTCGGAGC3'	5'GCAGGTCCCTGATGTAGTCG3'
Id2	5'CTCCAAGCTCAAGGAACTGG3'	5'ATTCAGATGCCTGCAAGGAC3'
Id3	5'TTAGCCAGGTGGAAATCCTG3'	5'TCAGTGGCAAAAGCTCCTCT3'
Rgs6	5'TCTCTCCAAAATCCCCAGTG3'	5'TTGCTTTGTTCTGCATCGTC3'
Smad6	5'ACGGTGACCTGCTGTCTCTT3'	5'AGCGAGTACGTGACCGTCTT3'
Gdf9	5'GATGTGACCTCCCTCCTTCA3'	5'GATGCTGTAAAGGCCTCCAG3'
Hesx1	5'ACAGACCCTGGACAGACACC3'	5'GTCAATGCCAGGGTAGCAGT3'
Tbc1d10a	5'ACCCCAAGTGGCTAGATGTG3'	5'AGCCAGGCAGGTACTTCTCA3'
Atp2c2	5'CACTCTGACAGCCAACGAAA3'	5'GCAGTAGGACTTCTGCTGGG3'
Stk25	5'TGCACTGGACTTGCTGAAAC3'	5'TTGGGAATCAGGAACAGGAC3'
Camk4	5'AGCTGGTCACAGGAGGAGAA3'	5'GGGGTTCCACACACTGTCTT3'
Calb1	5'GACGGAAGTGGTTACCTGGA3'	5'TTCCTCGCAGGACTTCAGTT3'

Chapter 4

In Chapter 3, I identified inhibitors of DNA binding 2 and 3 (*Id2* and *Id3*) as BMP2 target genes required for BMP2 and activin A to synergistically stimulate the *Fshb* reporter. Expression of the *Id*s, especially *Id1*, is often used as a marker of BMP activity. The mechanisms through which BMPs stimulate *Id1* expression have been described [377, 475-483]. Conversely, the exact signaling cascade and response elements required for BMP-induced *Id2* and *Id3* expression have not yet been completely characterized. In Chapter 4, I determined the mechanism by which BMP2 regulates *Id3* expression in gonadotropes. Additionally, I showed that this mechanism is conserved in fibroblasts and likely applies to the regulation of the human *ID3* gene expression as well.

Title: Mechanisms of bone morphogenetic protein 2 (BMP2) stimulated inhibitor of DNA binding 3 (Id3) transcription

Abbreviated title: BMP2 regulation of Id3 expression

Authors: Catherine C. Ho[1], Xiang Zhou[1], Yuji Mishina[2], and Daniel J. Bernard[1]

Affiliation: [1]Department of Pharmacology and Therapeutics, McGill University;
[2]Biologic and Materials Sciences, University of Michigan

Corresponding author:
Daniel J. Bernard, Ph.D.
Department of Pharmacology and Therapeutics
McGill University
3655 Promenade Sir William Osler
Montreal, Quebec (Canada)
H3G 1Y6
Ph: 514-398-2525
Fx: 514-398-6705
Email: daniel.bernard@mcgill.ca

Keywords: BMP, FSH, Id, BMRP1A, BMPR2, SMAD1/5

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Abstract

Bone morphogenetic protein 2 (BMP) stimulates expression of the inhibitors of DNA binding (Id) 1, 2, and 3 in a variety of cell types. Here, we examined mechanisms mediating BMP2-stimulated *Id3* transcription in murine gonadotropes. Using a combination of quantitative RT-PCR, promoter-reporter analyses, over-expression, and RNA interference approaches, we demonstrate that BMP2 signals via the BMPR2 and BMPR1A (ALK3) receptors and intracellular signaling proteins SMADs 1 and 5 to stimulate *Id3* transcription. We further define a novel 6-bp *cis*-element mediating BMP2- and SMAD-dependent transcription, though this site does not appear to bind SMADs directly. A specific DNA binding protein complex binds to this element, but its constituent protein(s) remain undetermined. Recently, a more distal enhancer was shown to mediate BMP4-induction of the human *ID3* gene in ovarian cancer cells. This enhancer is conserved in the murine gene and we demonstrate its role in BMP2-induced *Id3* promoter activity in gonadotropes. Conversely, the proximal *cis*-element defined here is also conserved in human *ID3* and we demonstrate its functional role in BMP2-induction of *ID3* transcription. Finally, we show that the two regulatory elements also mediate BMP2-induction of *Id3* promoter activity in murine fibroblasts. Collectively, we have defined a general mechanism whereby BMP2 regulates *Id3/ID3* transcription in different cell types and in different species.

Introduction

Bone morphogenetic proteins (BMPs) were originally identified as factors that induce ectopic bone and cartilage formation when implanted into muscular tissue [350]. BMPs are now known to play diverse roles, for example, in osteoblast and chondrocyte differentiation, tooth development, kidney development, skin and hair development, myogenic differentiation, neural cell differentiation, and vascular homeostasis [223]. Over 20 BMP family members, all part of the larger transforming growth factor β (TGFβ) superfamily, have been identified and characterized [223]. Although BMPs exhibit highly conserved structures, they can be classified into several subgroups based on their structural homology. For example, BMP2 and BMP4 are highly similar and form one subgroup [228, 240]. BMP2/4 signaling is initiated by binding to BMP type I serine/threonine receptors, ACVR1, BMPR1A and/or BMPR1B [436]. Type II receptors, such as BMPR2, are then recruited into the complex and phosphorylate the type I receptors [374, 435]. BMPs may also bind preassembled type I/type II receptor complexes [370-371]. BMP family members show some promiscuity in their receptor binding. For example, BMP2/4 preferentially signal through the type II receptor, BMPR2, but can use ACVR2 in its absence [368]. The activated type I receptors phosphorylate intracellular signaling proteins, the most thoroughly characterized of which are the receptor-regulated SMADs (or R-SMADs), SMADs 1, 5, and 8. Once phosphorylated, R-SMADs form heteromeric complexes with the co-regulatory SMAD (SMAD4) and accumulate in the nucleus. SMADs then regulate target gene transcription by directly binding to DNA and interacting with different transcriptional co-activators or co-repressors [250, 374-375]. The amino-terminal Mad homology 1 (MH1) domains of SMAD1/5/8 mediate their binding to GC-rich BMP response elements (BRE) in target genes [269].

The inhibitors of DNA binding (Id) are well-characterized BMP response genes in a variety of cell types [223, 381-384, 387-388, 484-485]. Four Id sub-types (Id1-4), which exhibit similar, but not identical biological activities [381], have been identified. Ids belong to the helix-loop-helix (HLH) family of transcriptional regulators. Unlike other HLH proteins, which can bind E-box *cis*-elements as homo- or hetero-dimers, Ids lack the basic amino acid domain necessary for DNA binding. Ids are instead conventionally thought to block transcriptional activity of bHLH proteins (such as Mash1, OLIG, NeruoD, and MyoD bHLH) by forming DNA-binding-deficient hetero-dimers [384, 388, 484-485].

Id proteins have been implicated as effectors of BMP signaling in a variety of cellular systems and have a role in neurogenesis, angiogenesis, and bone formation [381-384, 387-388, 484-485]. Recently, we and others reported that BMPs are expressed in LβT2 cells, an immortalized murine gonadotrope cell line, and in adult murine pituitary. In the former, BMP2 can stimulate

follicle-stimulating hormone β subunit (*Fshb*) gene expression alone and in synergy with activins [282, 401, 405]. We have demonstrated in Chapter 3 that BMP2 also induces *Id1*, *2*, and *3* mRNA expression in these cells and we demonstrated that BMP2 synergistically stimulates *Fshb* transcription with activins, at least in part, through the combined actions of Ids 2 and/or 3 and SMAD3, a major effector of activin signaling [297]. Here, we defined part of the mechanism whereby BMP2 regulates *Id3* transcription; ultimately providing a more complete understanding of BMP regulated *Fshb* expression.

Materials and Methods

Reagents

Human recombinant (rh-) BMP2 and activin A were purchased from R&D Systems (Minneapolis, MN, USA). Gentamycin, 1X phosphate buffered saline (PBS), and Dulbecco's Modified Eagle medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate were purchased from Wisent (St-Bruno, Quebec, Canada). Random primers, MMLV-reverse transcriptase, RNasin, and deoxynucleotide triphosphates (dNTPs), and 1X Passive Lysis Buffer (PLB) were from Promega (Madison, WI, USA). Protease inhibitor tablets (CompleteMini), and Expand Long Template PCR System were purchased from Roche (Mississauga, ON, Canada). Aprotinin, leupeptin, pepstatin, phenylmethylsulphonylfluoride (PMSF), SB431542, EZview Red ANTI-HA M2 Affinity Gel (Cat. # E6779), mouse monoclonal HA (#H9658), mouse monoclonal β-actin (#A5441), rabbit monoclonal FLAG (#F3165) antibody, cycloheximide, actinomycin D, pancreatin, and collagenase were from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Bio-Rad (Hercules, California, USA) and enhanced chemiluminescence (ECL) Plus reagents were from GE Healthcare (Piscataway, New Jersey, USA). Lipofectamine/Plus, Lipofectamine 2000, Media 199 (M199), Hanks' Balanced Salt Solution (HBSS), TRIzol Reagent, and SYBRgreen Supermix for qPCR were from Invitrogen (Burlington, ON, Canada). Oligonucleotides were purchased from IDT (Coralville, IA, USA). ^{32}P-ATP was from Perkin Elmer (Boston, MA, USA). Short-interfering (si) RNAs were purchased from Dharmacon (Lafayette, Colorado, USA): Control (Cat. # D-001210-05), *Acvr1* (Cat. # D-042047-01); *Bmpr1a* (Cat. # D-040598-01); *Bmpr1b* (Cat. # D-051071-01); *Smad1* (Cat. # D-055762-01 and D-055762-02); *Smad5* (Cat. # D-057015-01); *Smad8* (Cat. # D-046344-01 and D-046344-02); *Acvr2* (Cat. # D-040676–01), *Acvr2b* (Cat. # D-040629–02), and *Bmpr2* (Cat.# D-040599–01). Formaldehyde (37%) was from Fisher Scientific (Ottawa, ON, Canada). ChampionChIP One-Day kit was purchased from SABiosciences [distributed by Cedarlane; Burlington, ON, Canada]. Anti-SMAD1 (Cat# Sc-7965x)

was from Santa Cruz (Santa Cruz, CA, USA) and normal mouse IgG (Cat. # 12-371) was from Millipore [distributed by Cedarlane].

Constructs

The expression constructs for rat ACVR1-HA, human FLAG-SMAD1, murine FLAG-SMAD4, and rat SMAD5 were provided by Dr. Teresa Woodruff (Northwestern University, Chicago, IL, USA). The latter was sub-cloned into a pcDNA3.0 vector bearing an N-terminal FLAG tag. Human BMPR1A-HA (Q233D) and mouse BMPR1B-HA (Q203D) were provided by Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan). Rat myc-SMAD8 and murine FLAG-SMAD5 were provided by Dr. C.H. Heldin (Ludwig Institute for Cancer Research, Sweden) and Dr. T. Watanabe (Tokyo University, Tokyo, Japan), respectively. The rat FLAG-SMAD8 construct was generated in-house by PCR using MYC-SMAD8 as template. The murine -1561/+15 *Id2*-luciferase and -886/+15 *Id3*-luciferase promoter-reporters were provided by Dr. Yoshifumi Yokota (University of Fukui, Fukui, Japan) [486] and Dr. Robert W. Lim (University of Missouri-Columbia, Columbia, USA) [487], respectively, and their 5'deletions generated by PCR as previously described [297] (see Supplemental Table S4.1 for primers). The murine *-3740/+24 Id3*-luciferase reporter was generated from wild-type C57BL6/J mouse genomic DNA using the Expand Long Template PCR kit (Roche) and the primers indicated in Supplementary Table S4.1 and ligated into the *Mlu*I and *Xho*I sites in pGL3-Basic (Promega). The human *ID3* promoter-reporter constructs (-4104/+402, -1927/+402, -653/+402, and +36/+402) and parental pGL2-Basic vector were provided by Dr. Trevor Shepherd (University of Western Ontario, ON, Canada). Note that the numbering of the constructs has been modified here relative to that reported in [379]. These changes were made based on our sequencing of the ends of the constructs and comparing them to the human *ID3* mRNA and genomic sequences described in GenBank acc. #NM_002167 and NC_000001.10, respectively. Site-directed mutagenesis of the murine *Id3* and human *ID3* promoter-reporters as well as of the SMAD1 and SMAD8 siRNA-sensitive expression constructs was performed using the Stratagene QuikChange protocol (Agilent Technologies, Mississauga, ON, Canada). The GATA4-HA and dominant-negative GATA4 constructs were provided by Dr. Robert Viger (Université Laval, Québec City, Québec, Canada).

Cell culture and transfections

Immortalized murine gonadotrope LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego, CA, USA) and were cultured in 10% FBS/DMEM and 4 μg/ml gentamycin as described previously [297]. For luciferase assays, cells were plated in 24-well plates

(2.5 x 10^5 cells per well) approximately 36 h prior to transfection. Cells were transfected with 450 ng reporter/well using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). Twenty-four h after transfection, cells were washed in 1X PBS and then treated with ~1 nM (25 ng/ml) BMP2 plus 10 μM SB431542 in DMEM or with 10 μM SB431542 in DMEM alone (control) for the indicated times. The ALK4/5/7 inhibitor SB431542 [349] was included to block any potential effects of endogenous activin B. However, it should be noted that inclusion of the inhibitor was not required to observe BMP2-induced *Id2* or *Id3* expression, and that any effects of activins are likely to be modest and mediated via mechanisms distinct from those underlying BMP2 responses (data not shown). In time-course experiments, the introduction of ligand was staggered so that protein lysates from different treatment groups were collected at the same time. In over-expression experiments, LβT2 cells cultured in 24-well plates were transfected with the reporter as described above plus 100 ng of each receptor or SMAD expression vector (or empty vector for controls) per well. Cells were changed to serum-free media and lysates collected 24 h later. In RNA interference (RNAi) experiments, siRNAs in 1X siRNA buffer (20 mM KCl, 6 mM HEPES-pH7.5, and 0.2 mM $MgCl_2$) were transfected at a final concentration of 5 nM. Resulting data were normalized to cells transfected with the control siRNA. Twenty-four h after transfection, cells were washed in 1X PBS and treated with BMP2 and SB431542 for 24 h as described above. Lysates were collected 24 h later to measure luciferase activity. LβT2 cells plated in 6-well plates (1 x 10^6 cells per well) approximately 48 h prior to treatment were washed in serum-free DMEM and then treated with 25 ng/ml BMP2 in DMEM with or without 5 μg/ml cycloheximide or 5 μg/ml actinomycin D for 1 h. Cells were washed with 1X PBS and total RNA was collected with TRIzol (Invitrogen) for qPCR analysis following the manufacturer's instructions. Nuclear extracts were collected from LβT2 cells cultured from 10 cm plates as previously described [296] and used for gel shift experiments. For chromatin immunopreciptitation (ChIP) analyses [see details below], approximately 5x10^6 LβT2 cells cultured in 10-cm dishes for approximately 48 h were washed with 1X PBS and treated for 1 h with ~2 nM (50 ng/ml) BMP2 plus 10 μM SB431542 in DMEM. Cells were fixed and harvested following the manufacturer's instructions (SABiosciences).

CHO and NIH3T3 cells were obtained from Dr. Patricia Morris (Population Council, New York, NY). CHO cells cultured in F-12/DMEM containing 10% FBS and 4 μg/ml gentamycin in 6-well plates were transfected when 70–80% confluent with 100 ng of the indicated FLAG-SMAD 1, 5, or 8 expression constructs and 10 nM siRNA using Lipofectamine/Plus for 6 h and then changed to growth media. Cell lysates were then harvested the following day for western blot analysis. CHO cells grown in 10-cm dishes were transfected using Lipofectamine/Plus and 4 μg of the indicated HA-tagged GATA4 and FLAG-tagged SMAD1 expression vectors. Protein lysates were harvested the

following day for use in immunoprecipitation analyses [see below]. NIH3T3 cells used for luciferase assays were cultured at 10^5 cells per well in 10% FBS/DMEM and 4 µg/ml gentamycin. Transfection protocols were identical to those used for the LβT2 cells.

Luciferase assays

Cells were washed with 1X PBS and lysed in 1X PLB. Luciferase assays were performed on an Orion II microplate luminometer (Berthold detection systems, Oak Ridge, TN, USA) using standard reagents [297]. All treatments were performed in duplicate or triplicate as described in figure legends. Data are represented as means of means (+SEM or SD) from three or more independent experiments.

Reverse transcription and qPCR

Reverse transcription was performed on 1-2 µg of total RNA as previously described [282]. qRT-PCR was performed on the resulting cDNA using the SYBRgreen Supermix following the manufacturer's instructions with a Corbett Rotorgene 6000 qPCR machine (Corbett Life Science). As results from replicate qPCR experiments were qualitatively similar, but sometimes quantitatively different (in terms of fold effects), we presented data from one representative experiment out of three individual experiments (Figure 4.1 and 4.3C). The remaining two replicates in each experiment were presented as supplementary Figures S4.1 and S4.2. Expression of target genes was normalized relative to ribosomal protein L19 (*Rpl19*) in the same sample and presented relative to the no ligand control. Sequences of the qPCR primers for *Rpl19*, *Id2*, *Id3*, and *Bmpr1a* genes are shown in Supplementary Table S4.1.

Primary pituitary cultures and adenoviral infection

Male *Bmpr1a*$^{flox/flox}$ mice [488] were sacrificed at 8 weeks of age in accordance with institutional and federal guidelines. Pituitaries were extracted, quartered using scalpel blades, and digested with collagenase for 2 h in a 36°C shaking water bath. Cells were collected by centrifugation at 1000 x g for 5 min, resuspended in calcium free HBSS and further digested win 2X pancreatin for 15 min. Cells were collected by centrifugation, washed in complete M199 media four times, and then passed through a 40 µm filter cloth to remove cell debris. 10^5 cells were plated per well in 96-well dishes. Cells were cultured in 10% FBS/M199 medium for 36 h before infection with adenovirus expressing GFP or Cre-IRES-GFP (provided by Dr. Derek Boerboom, Université de Montréal,

Canada). Infection was verified 24 h later using Zeiss Axio Observer A1 fluorescent inverted microscope to detect GFP expression. Cultures were then pre-treated with 10 μM SB431542 in 10% FBS/M199 for 24 h. Cells were then washed with 1X PBS and treated (in the absence of SB431542) with 25 ng/ml activin A or 50 ng/ml BMP2 in 2% FBS/M199 for 24 h. Total RNA was extracted with TRIzol (Invitrogen) following the manufacturer's instructions for qPCR analysis. Genomic DNA was also prepared from the same extracts and subjected to genotyping analysis by PCR (to confirm recombination) using primer sets Fx1/4 or Fx2/4 shown in Table S4.1 with an annealing temperature of 55°C for 35 cycles.

Immunoprecipitation

CHO cells were washed with 1X PBS and whole cell protein extracts (WCE) prepared with lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and centrifuged at 10,000 x rpm for 15 min at 4°C to remove insoluble material. WCEs were subjected to immunoprecipitation using EZview Red ANTI-HA M2 Affinity Gel following the manufacturer's instructions. In brief, WCEs were incubated with the affinity gel on a rotating platform overnight at 4°C to allow binding of HA-GATA4 to the ANTI-HA affinity gel. The affinity gel was then incubated in 1X HA peptide (Cat. # I2149) solution on a rotating platform for 45 min at 4°C to elute gel-bound proteins. The eluted proteins were then analyzed by western blot using anti-FLAG and anti-HA [see below].

Western blotting

Cells were washed with 1X PBS and whole cell protein extracts (WCE) prepared with 1X RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 0.01 M sodium phosphate pH 6.8, 2 mM EDTA, 50 mM sodium fluoride, and CompleteMini Protease Inhibitor Cocktail Tablets) and centrifuged at 13,000 × g for 0.5 h at 4ºC to remove insoluble material. WCEs were subjected to western blot analyses as previously described [297]. Briefly, equivalent amounts of protein were separated by SDS-PAGE and transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Blots were probed with the indicated antibodies using standard techniques [293, 297].

Electrophoretic mobility shift assays

Gel shift experiments were performed as described [489], with minor alterations to the protocol. Briefly, nuclear protein concentrations were determined by Bradford assay (BioRad). Nuclear proteins (3–5 µg) were incubated for 20 min at room temperature with 50 fmol of ^{32}P-ATP end-labeled double-stranded DNA probes corresponding to the indicated fragments (see Figs. 3.5B, 3.5C, S3.6A) of the murine *Id3* promoter in binding buffer (25 mM HEPES pH 7.2, 150 mM KCl, 5 mM dithiothreitol, 10% glycerol). Five hundred ng salmon sperm DNA or 0.5–1 µg poly(dI).poly(dC) was used in the binding buffer as non-specific competitor. In competition experiments, reactions were assembled at room temperature and incubated for 10 min with 100-fold molar excess unlabeled (cold) competitor probes prior to the addition of the radio-labeled probe. Reactions were then run on 5% polyacrylamide gels (44:0.8 acrylamide:bis-acrylamide) in 40 mM Tris–HCl/195 mM glycine (pH 8.5) at 200 volts for 3–5 h at 4 °C. Gels were dried and exposed to X-ray film (Kodak).

Chromatin immunoprecipitation (ChIP) assays

Approximately 4-6 x10^6 LβT2 cells per 10-cm culture dish were harvested for each experiment according to the manufacturer's guidelines (SABiosciences). In brief, crosslinking was performed for 10 min at room temperature with 1% formaldehyde diluted in 1X PBS. Cells were then quenched in 1X glycine for 5 min, washed twice with cold 1X PBS, and harvested with 1X PBS containing protease inhibitor cocktail using a rubber policeman. Cells were then collected by centrifugation and lysed with the provided lysis buffer. Cell contents (DNA/protein complexes) were sonicated using a Misonix Sonicator 3000 (Mandel, Guelph, ON, Canada) to obtain chromatin fragments around 750 bp (35 sec; 5 sec ON, 10 sec OFF). Lysates were collected by centrifugation at high speed for 10 min at 4°C. Two-hundred µl aliquots of sheared chromatin were subjected to pre-clearing using Protein A beads for 1 h at 4°C. Ten µl aliquots of each sample were removed to be used as input control. The remaining chromatin fractions were subjected to immunoprecipitation using 4 µg of SMAD1 Ab or 4 µg of the control normal mouse IgG overnight at 4°C with rotation followed by 1 h incubation with Protein A beads. Beads were washed with the provided wash buffers (1-4) in five sequential steps at room temperature. Reverse crosslinking was performed in a 45°C shaking water bath with 20 µg proteinase K diluted in the provided elution buffer for 30 min, followed by a 10 min incubation at 95°C. DNA purification was performed using the provided DNA spin columns and eluted with elution buffer. Quantitative real-time PCR was performed using 4-6 µl of the eluted DNA for 40 cycles using the SYBRgreen Supermix following the manufacturer's instructions with a Corbett Rotorgene 6000. Data were analyzed following SABiosciences ChIP

quantitative PCR analysis guidelines by normalizing against the input and samples immunoprecipitated with control IgG (equivalent to the ΔΔCt method). The resulting data represent the mean of three independent experiments (N=1 per experiment). Sequences of the qPCR primers for the various *Id3* promoter fragments are shown in Table S4.1.

Data analyses

In all cases, reporter assay data from three replicate experiments were highly similar. Therefore, means of treatment replicates within each experiment were calculated to generate a single value per treatment per experiment. The data from replicate (three or more) experiments were then used for statistical analyses. Figures shown for qPCR data are representative graphs, unless otherwise indicated; experiments were performed three times with results showing similar trends. Data are presented as fold-change from the control condition (no ligand and/or transfection with empty vector alone). Differences between means were compared using one-, two-, or three-way analyses of variance followed by post-hoc pair-wise comparison with Bonferroni adjustment where appropriate (Systat 10.2, Richmond, CA, USA). Bars or values with different symbols were statistically different, whereas those sharing symbols did not differ. Significance was assessed relative to $p < 0.05$.

Results

BMP2 stimulates *Id2* and *Id3* transcription in gonadotropes

We previously reported in Chapter 3 that BMP2 stimulates increases in *Id1*, *Id2*, and *Id3* mRNA levels in LβT2 cells. Here, we established that the increases in *Id2* and *Id3* mRNA levels reflect direct transcriptional responses. BMP2-stimulated *Id2* and *Id3* mRNA expression was completely blocked by pre-treatment with the transcriptional inhibitor actinomycin D (Figure 4.1 and Supplementary Figure S4.1). In contrast, pre-treatment with the translational inhibitor cycloheximide did not block BMP2-stimulated *Id2* or *Id3* expression. These data suggest that *Id2* and *Id3* are BMP2 immediate-early response genes in LβT2 cells.

The murine *Id3* promoter is time- and dose-dependently stimulated by BMP2

To uncover mechanisms mediating BMP2-induced *Id2* and *Id3* transcription, we turned to promoter-reporter assays. When transfected into LβT2 cells, the murine -1561/+15 *Id2*-luciferase reporter [486] was unresponsive to BMP2 (data not shown). It is possible that critical regulatory sequence was not present within the promoter fragments used. In contrast, a murine -886/+15 *Id3*-

luciferase reporter [487] was time (Figure 4.2A) and concentration-dependently stimulated by BMP2 (Figure 4.2B). Induction of reporter activity (Fig. 4.2A) showed slower kinetics than did induction of the endogenous gene (Fig. 4.1); however, differences in both the nature and sensitivity of the assays likely explains (at least in part) this apparent discrepancy. The empty vector, pGL3-Basic, was not regulated by BMP2. It should be noted that the promoter itself, in the absence of BMP2, conferred significant basal reporter activity (compare the open and closed symbols at time 0 or in the absence of BMP2 in Figs. 4.2A and 4.2B). Based on these observations, subsequent reporter assays were limited to the *Id3* promoter and treatments were performed with 25 ng/ml BMP2 for 24 h. Next, 5' deletions were used to define the minimally responsive reporter. Truncations from -886 to -568 did not significantly modify fold BMP2 induction (Figure 4.2C and data not shown). However, further deletion to -502 completely abrogated the BMP2 response, while maintaining basal reporter activity. The minimal promoter-reporter, -568/+15 *Id3*-luc, was used in subsequent experiments.

BMP2 signals preferentially through BMPR1A and BMPR2 to regulate *Id3* promoter activity

To determine the relevant signaling receptor(s) in this system, we used siRNAs to knock down endogenous expression of each of the type I receptors for BMP2 in LβT2 cells, and measured the fold BMP2 stimulation of the minimal *Id3* promoter-reporter. All the relevant BMP type I receptors are expressed in these cells [282, 405, 490]. The *Bmpr1a* siRNA abolished BMP2-stimulated *Id3* promoter activity, whereas the *Acvr1* and *Bmpr1b* siRNAs were without effect (Figure 4.3A). The sequence specificity and efficiency of all of the siRNAs were previously validated in Chapter 2. These data suggests that BMP2 preferentially signals through the type I receptor, BMPR1A, to stimulate *Id3* expression in LβT2 cells. In complementary assays, the *Id3* promoter was transfected together with different constitutively active type I receptors (Gln→Asp) [491]. *Id3* promoter activity was stimulated significantly by BMPR1A-QD, less so with BMPR1B-QD, and not at all by ACVR1-QD (Figure 4.3B). This is consistent with the idea that BMPR1A-mediated signaling induces *Id3* transcription.

To determine whether BMPR1A is important for BMP2-induced *Id3* expression in a more physiological context, we prepared primary pituitary cultures from floxed *Bmpr1a* mice [488]. Cells were infected with adenoviruses expressing GFP (control) or Cre recombinase and GFP (from a bi-cistronic mRNA). The cultures were then treated with activin A or BMP2 for 24 h. In control cells, BMP2 (but not activin A) stimulated an increase in *Id3* mRNA expression (Figure 4.3C and Figure S4.2), consistent with our results from LβT2 cells in Chapter 3 (Figure 4.1, and data not shown). In cells transduced with Cre expressing virus, the BMP2 effect was abrogated (Figure 4.3C and Figure

S4.2). Analysis of genomic DNA and RNA confirmed recombination of the *Bmpr1a* gene and the associated depletion of *Bmpr1a* mRNA in these cells (Figure S4.3). These data suggest that BMP2 signals through BMPR1A to stimulate *Id3* transcription in murine pituitary cells.

Having established BMPR1A as the relevant type I receptor, we next sought to determine with which type II receptor it cooperates to mediate BMP2 activity. Knock down of *Acvr2* or *Acvr2b* with specific siRNAs did not affect BMP2-induced *Id3* transcription, whereas depletion of *Bmpr2* significantly diminished the BMP2 response (Figure 4.3D). Collectively, these data suggest that BMP2 preferentially signals through BMPR1A and BMPR2 to stimulate *Id3* expression in gonadotropes.

BMP2 signals through SMAD1/5 to regulate *Id3* promoter activity

To determine the relevant signal transducers in this system, we used siRNAs to knockdown expression of each of the BMP R-SMADs. Depletion of endogenous SMAD1 or SMAD5 significantly reduced BMP2-stimulated *Id3* promoter-reporter activity, whereas SMAD8 knockdown had little or no effect (Figure 4.4A). To confirm the efficacy of the siRNAs, we examined the effects of the siRNAs on SMAD1/5/8 protein expression. Under our assay conditions, transfection efficiency of LβT2 cells is insufficient to obtain an accurate measure of the extent of RNAi-mediated knockdown of mRNA/protein expression on a per cell basis [297]. Therefore, to validate the *Smad5* siRNA, we over-expressed a murine FLAG-SMAD5 construct, which is sensitive to the siRNA, and a rat FLAG-SMAD5 construct, which is resistant to the siRNA, in CHO cells in the presence or absence of the *Smad5* siRNA (Figure S4.4). We did not have a murine SMAD1 expression vector to validate the *Smad1* siRNA. Therefore, we used an available human-SMAD1 construct and modified its nucleotide sequence to make it sensitive to the murine *Smad1* siRNA. Two different targeting siRNAs were used; therefore, two siRNA resistant constructs were generated. This was similarly done with an available rat SMAD8 construct. The siRNA-resistant and -sensitive constructs were expressed in CHO cells in the presence or absence of their respective *Smad* siRNAs. Western blot analysis confirmed that each siRNA potently inhibited protein expression of its siRNA-sensitive target, but not the siRNA-resistant form of the construct. BMP2-induced *Id3* reporter activity, which was inhibited by the S*mad5* siRNA, was rescued by over-expression of an siRNA-resistant rat SMAD5 (Figure S4.5). In complementary assays, the *Id3* promoter-reporter was transfected together with different R-SMADs alone or in combination with SMAD4, and then treated with BMP2 (Figure 4.4B). BMP2 induction of *Id3* promoter activity was potentiated most robustly when SMAD4 was co-

expressed with SMAD1 or SMAD5. Together, these data suggest that BMP2 signals through complexes of SMAD1/4 and/or SMAD5/4 to regulate murine *Id3* transcription in gonadotrope cells.

Identification of a novel BMP2 response element in the murine *Id3* promoter

Above, we defined a BMP2 responsive region of the murine *Id3* promoter in the interval between -568 and -502 relative to the start of transcription (Figure 4.2C). Using additional 5' deletions, we further defined two BMP2 responsive regions; one between -568/-548 and the other between -528/-502 (Figure 4.5A). Several nuclear proteins from LβT2 cells could bind within these intervals as revealed by electrophoretic mobility shift assays (EMSAs) (Figure 4.5B, see lanes 2 and 12). Specificity of complex binding was demonstrated by co-incubation with unlabeled competitor probes (compare lanes 2 and 3; and lanes 12 and 15). Complex formation was unaffected by BMP2 treatment (data not shown).

To identify the specific base pairs (bp) mediating protein complex binding, 3-bp scanning mutations were first introduced into -528/-502 competitor probes (Figure 4.5C). All four complexes (labeled A-D at left) were competed by the wild-type probe (lane 13). In contrast, probes bearing mutations 4 and 5 (lanes 6 and 7) failed to compete for complex D, suggesting that bp -519/-514 mediate binding of the protein(s) in this complex. Results with the other competitors suggest that the protein(s) in complex C bind to bp -525/-514 (lanes 4-7), in complex B to bp -525/-517 (lanes 4-6), and in complex A to bp -516/-511 (lanes 7 and 8). Mut2 through Mut6 were next individually introduced into the murine -568/+15 *Id3* promoter-reporter. None of the mutations affected basal reporter activity in LβT2 cells. In contrast, Mut4 and Mut5 both impaired BMP2-stimulated promoter activity (Figure 4.5D). These data suggest that binding of the protein(s) in complex D [though not BMP2-regulated in these assays] may be required for BMP2-induced promoter activity. The same approach was used to identify the base pairs required for protein complex formation within the -568/-542 interval; however, the competition data were less clear (Figure S4.6A) and introduction of the corresponding mutations into the *Id3* promoter did not significantly impair reporter activity (Figure S4.6B).

Supershift experiments in EMSA (data not shown), DNA affinity pull-down (DNAP; data not shown), and chromatin immunoprecipitation analyses (ChIP; Figure S4.7A) all failed to demonstrate SMAD1/5 binding to this element. Nonetheless, both Mut4 (Figure 4.5E) and Mut5 (data not shown) inhibited SMAD5/4 induction of promoter activity. Based on *in silico* analyses, the base pairs mediating complex D binding resembled a GATA factor binding site. Furthermore, both super-shift and DNAP analyses suggested that one or more GATA proteins could bind this element (data not

shown). However, transfection of a dominant-negative GATA4 had no impact on basal or BMP2-regulated promoter activity (data not shown) and we were unable to demonstrate an interaction between SMAD1 or 5 and GATA4, despite replicating previous work showing that SMAD3 and GATA4 interact [492] (Figure S4.7B and data not shown). Although our data suggest that GATA proteins may be part of complex D, functional data did not corroborate a role for them, so the identity of the components of complex D mediating BMP responsiveness is unclear at present.

Conservation of the BMP2 response element in the human *ID3* promoter

In the course of our investigations, a mechanism for BMP4-stimulated human *ID3* expression in ovarian cancer cells (CaOV3) was described [379]. In that report, an enhancer was described upstream of the promoter region we investigated here in mouse. We obtained the reporter constructs used in [379] and examined their BMP2 induction in LβT2 cells (Figure 4.6A). The full-length human reporter (-4104/+402) was robustly stimulated by BMP2 and the fold-induction (though not basal activity) was diminished by truncation to -1927, which removes the distal enhancer. However, the -1927 and -653 reporters were still induced by BMP2 and the BMP2 response was lost only following further truncation to +36. The 6-bp (-519/-514) mediating BMP2 induction of the murine *Id3* promoter (defined above) are perfectly conserved in the human gene (-188/-183) (Figure S4.8). To determine if these base pairs also play a role in BMP2 induction of the human *ID3* promoter, we introduced mutations comparable to Mut4 and Mut5 in the murine *Id3* promoter into the minimal *hID3* promoter-reporter construct (-653/+402 *ID3*-luc) (Figure 4.6B). Similar to what we observed with the murine *Id3* promoter, both mutations significantly impaired BMP2 induction of human *ID3* promoter activity.

The BRE in the human distal enhancer is conserved in the murine *Id3* promoter

In silico analyses suggested that the upstream enhancer in the human *ID3* gene might be conserved in murine *Id3* (Figure S4.8). As the relevant sequence maps approximately 3.6 kb upstream of the transcriptional start site in the murine *Id3* gene, it was not represented in the reporter constructs used in our initial analyses. We therefore generated a larger murine *Id3* promoter-reporter and observed that it was more strongly induced by BMP2 in LβT2 cells than was the -568/+15 reporter used above (Figure 4.7A). The analysis of the human gene revealed a BMP response element (BRE) within a distal enhancer (-2632/-2625) that when mutated (TGGCGCC→ TGGTGCT) greatly reduced the fold BMP4 response [379]. We identified the same sequence in the murine gene (-3283/-3276) (Figure S4.8) and observed through ChIP analysis that BMP2 stimulated recruitment of

SMAD1 to this region of the endogenous *Id3* gene in LβT2 cells (Figure S4.7A). Mutation of the distal BRE in the longer murine *Id3* reporter greatly reduced, but did not abolish, BMP2 induction (Figure 4.7B). Introduction of Mut4 or Mut5 in the context of the longer reporter also reduced the BMP2 response, though to a lesser extent than the mutation to the distal BRE (Figure 4.7B). Importantly, the BRE and Mut4 mutations in combination almost completely blocked BMP2-stimulated reporter activity, demonstrating that both the proximal and distal BREs work in concert to mediate the BMP2 response.

The mechanism of BMP2-regulated *Id3* transcription is conserved in fibroblasts

The above analyses were conducted exclusively in LβT2 or primary pituitary cells. To determine whether or not the described mechanisms were cell-type specific, we examined BMP2-stimulated promoter activity in NIH3T3 cells, the first cell type in which BMPs were shown to stimulate *Id3* expression [493-494]. Both the 0.57 and 3.7 kb murine *Id3* reporters were induced by BMP2 in NIH3T3 cells (Figure 4.7C). As in LβT2 cells, mutations made simultaneously to the distal and the proximal BREs abrogated BMP2 induction (Figure 4.7D), demonstrating conservation of the mechanism.

Discussion

In the current study, we determined that BMP2 signals preferentially through the type I receptor, BMPR1A, and type II receptor, BMPR2, to regulate *Id3* transcription in LβT2 cells. BMPR1A also mediates BMP2-induced *Id3* expression in primary pituitary cultures. Further, we showed that the BMP response in LβT2 cells is mediated through SMAD1/5 and requires a conserved promoter element (-519/-514 in mouse; -188/-183 in human). Finally, we found that *Id3* transcription is further enhanced by a distal BRE first described in the human ID3 gene [379].

*Id*s are well-known BMP responsive genes. A mechanism for BMP-stimulated *Id1/ID1* expression has been described. Selective deletion of *Bmpr1a* in murine endocardium suggests that BMPs preferentially signal through BMPR1A to stimulate *Id1* expression [476]. Depletion of *Bmpr2* in cultured cells by siRNA or via Cre-mediated recombination in mice suggests that BMPR2 plays a crucial role in the sustained induction of *Id1* expression by BMP4 in vascular smooth muscle cells [480, 482]. The data reported here similarly define BMPR1A and BMPR2 as transducers of the BMP2 signal to the *Id3* promoter in gonadotrope cells. *Id3* mRNA expression is abolished in endocardium of conditional *Bmpr1a* knockout mice [476], suggesting that BMPR1A is likely necessary for *Id3* expression in a variety of cell types. The type I receptors ACVR1 and BMPR1A are

widely expressed in various cell types, whereas BMPR1B shows more restricted expression [223, 228, 363-365]. It was previously described that BMPR1B is endogenously expressed in LβT2 cells [282, 409]. The structure of BMPR1A and BMPR1B are highly similar. Moreover, BMP2 and BMP4 bind to BMPR1A and BMPR1B with higher affinity than to ACVR1 [223, 228, 363]. It is therefore interesting that BMPR1A is uniquely required for BMP2 induction of *Id3* transcription in LβT2 cells, and that its loss cannot be compensated for by BMPR1B. Perhaps the latter is expressed at too low a level to functionally compensate [405, 408] or the two receptors may function distinctly in this context [454, 495-496].

BMPs induce *Id1/ID1* expression through the activation of SMADs 1, 5, and 4 [377, 475, 477-479, 481]. Our data similarly implicate these SMADs in BMP2 induction of murine *Id3* in gonadotropes whereas BMP4 induction of human *ID3* in vascular smooth muscle cells and ovarian cancer cells is also SMAD1/5/4-dependent [379, 497]. Interestingly, the loss of SMAD1 in gonadotropes cannot be fully compensated for by SMAD5, and vice versa, suggesting that the two SMADs may assume different roles in *Id3* regulation and/or that heteromers of SMAD1/5/4 may be most effective in stimulating *Id3* transcription.

Several BMP responsive elements have been described in the *Id1/ID1* promoter. One study identified a GC-rich region between -985/-957 of human *ID1* promoter as a necessary BRE [477]. A second study identified the same GC-rich element and three additional CAGAC boxes as *cis*-elements required for BMP2-mediated induction of a human *ID1* reporter. Both sites were observed to bind SMAD1/4 and are located between -1046/-863 of the *ID1* promoter [377]. In the murine *Id1* promoter, however, the BRE was localized between -1133/-1025 [483]. This region also contains a GC-rich BRE, specifically the GGCGCC palindrome, for binding SMAD5/4, and two CAGA(C) boxes for binding SMAD4. In contrast to the case for *Id1/ID1*, where there may be species diversity in BMP regulatory mechanisms, we identified a conserved BRE in the proximal murine *Id3* promoter that is physically and functionally conserved in the human *ID3* gene.

Specifically, we identified a 6-bp element critical for BMP2-mediated *Id3* transcription. Though a specific protein complex was shown to bind this site, its binding was BMP2-independent and its constituents remain undetermined. Furthermore, we were unable to demonstrate SMAD1 or SMAD5 as members of this complex, even though SMAD5/4 induction of *Id3* transcription is dependent upon this *cis*-element. Collectively, the data suggest that SMAD1/5 requires the promoter element at -519/-514 (hereafter proximal BRE) to mediate the BMP2 response and that SMADs may produce their effects via protein-protein interaction rather than via direct DNA binding.

Though we identified this novel BRE, AAGATA, in the proximal murine *Id3* promoter, Shepherd *et al.* recently reported that BMP4 stimulates human *ID3* transcription in ovarian cancer cells via a more distal BRE, TGGCGCC, in the human *ID3* promoter [379]. This observation led us to examine whether this latter element is also necessary for BMP2 induction of the murine *Id3* gene in gonadotropes. Indeed, the distal BRE is conserved in the murine *Id3* promoter and strongly contributes to BMP2 induction of both the human and murine *ID3*/*Id3* reporters. As indicated above, the proximal BRE in the murine *Id3* promoter is conserved in the proximal human *ID3* promoter and contributes to BMP2 induction of both the human and murine *ID3/Id3* reporters. When the two BREs were mutated in combination, the reduction in BMP2 activity was synergistic, suggesting that the proximal and distal BREs may cooperate in the regulation of human and murine *ID3*/*Id3* transcription. Shepherd *et al.* demonstrated the direct binding of SMAD1/5/4 to the distal BRE of the human *ID3* promoter; here we also show that SMAD1 binds to the distal BRE of the murine *Id3* promoter. At present, similar efforts have been unsuccessful in demonstrating SMAD1 binding to the proximal BRE.

BMPs regulate target gene expression through SMAD1/5/8 binding to GC-rich BREs, including GCCG elements [269, 498-500] and 'bipartite elements', which are composed of the consensus sequence TGGCGCC with so-called 'CAGAC boxes' found in close proximity [378]. These bipartite elements are conserved between many BMP target genes, including of all four of the *Id* genes [378]. The proximal and distal BREs are perfectly conserved across all of the examined mammalian *ID3*/*Id3* promoters, including human, mouse, rat, cow, chimpanzee, and dog (Figure S4.8). Furthermore, a conserved CAGAC box is found in close proximity to both the proximal and distal BREs in all cases; hence, both the proximal and distal BREs are potential bipartite elements. It has been proposed that BMP-regulated SMADs bind to the BRE site, whereas the CAGAC box mediates SMAD4 binding [378]. Indeed, BMP2 and BMP4 activity was significantly reduced when mutations were made to either the distal BRE site or its accompanying CAGAC box ([379] and data not shown). The CAGAC box within the proximal candidate bipartite element may also play a role in directing BMP2 responsiveness by facilitating the actions of SMAD1/5 on the BRE; however, we have not yet studied this possibility. In addition to the distal site, Shepherd *et al.* also identified another bipartite element within the second intron of the *ID3* gene. These two elements were found to independently and synergistically regulate BMP4 mediated *ID3* expression in ovarian cancer cells [379]. Here, we identified a potential bipartite element in the proximal *ID3/Id3* promoter, suggesting the possibility for a three-way interaction between the distal, proximal, and intronic elements to cooperatively regulate *ID3/Id3* expression. The intronic BRE identified in the human *ID3* promoter is also conserved in all the examined mammalian species (not shown); however, the closely associated

CAGAC box is only present in human and chimpanzee suggesting that conservation may be limited to primates.

BMP stimulated *Id3* expression was first identified in the NIH3T3 cells, a mouse embryonic fibroblast cell line [493-494]. We confirmed that both the distal and proximal BREs described here also mediate BMP2 induction of *Id3* transcription in these cells. This suggests that BMP2 likely regulates *Id3* expression through a mechanism common to most cell types, where the proximal and distal BREs act cooperatively to regulate *Id3* transcription.

Though we also demonstrated that BMP2 stimulates immediate-early induction of *Id2* transcription, we were unable to determine the underlying mechanisms because the murine -1561/+15 *Id2*-luciferase reporter available to us was unresponsive to BMP2. Recent data suggest that this likely stemmed from the absence of critical regulatory sequence in this reporter. In C2C12 cells, a longer *Id2* promoter-reporter (-3000/+80) was induced by BMP6 via a bipartite element at approximately -2.7 kb relative to the transcription start site [501]. Whether the same *cis*-element mediates BMP2 induction of *Id2* in gonadotrope cells remains to be determined.

In summary, we have determined relevant signaling components BMP2 employs to regulate *Id3* transcription. We have also identified a novel BMP2 response element in the proximal *ID3/Id3* promoter, which functions cooperatively with a distal element to regulate human and murine *ID3/Id3* expression. The mode of BMP2-mediated *Id3* expression we described here is likely a general mechanism conserved across cell types and mammalian species. Results from the present study may also contribute a more complete understanding of mechanisms controlling FSH synthesis as Id3 has been implicated in BMP2-induced *Fshb* transcription.

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Figure Legends

Figure 4.1. LβT2 cells seeded in 6-well plates were pre-treated with 5 µg/ml cycloheximide or actinomycin D for 15 min prior to 25 ng/ml BMP2 treatment for 1 h. Changes in *Id2* and *Id3* mRNA expression were measured by qRT-PCR. Data are normalized to the housekeeping gene, *Rpl19*, and presented as fold change in mRNA expression relative to untreated cells. The figure shown is representative of three replicate experiments (see Supplemental Fig. S1 for the other replicates). *Id2* and *Id3* mRNA transcripts were analyzed separately. Here and in subsequent figures, bars with different symbols were statistically different, whereas those sharing symbols did not differ.

Figure 4.2. A) LβT2 cells seeded in 24-well plates were transfected with murine -886/+15 *Id3*-luc and treated with 25 ng/ml BMP2 for 0, 4, 8, or 24 h in serum-free medium containing 10 µM SB431542. B) LβT2 cells seeded and transfected as above were treated with 0, 10, 25, or 50 ng/ml of BMP2 for 24 h in serum-free medium containing 10 µM SB431542. In both panels, treatments were performed in triplicate, the data reflect the mean (+SEM) luciferase activity of three independent experiments and are presented relative to the control group, in which the cells were transfected with the pGL3-Basic vector and no ligand was applied. C) LβT2 cells seeded in 24-well plates were transfected with the indicated 5' deletions of the murine *Id3* promoter-reporter and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 µM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to pGL3-Basic transfected cells in the absence of ligand. Values beside the bars represent the fold stimulation by BMP2 for each reporter.

Figure 4.3. A) LβT2 cells seeded in 24-well plates were transfected with murine -568/+15 *Id3*-luc and 5 nM of the control short interfering RNA (siRNA) or siRNAs for the indicated BMP type I receptors. Cells were then treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 µM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which control siRNA was transfected but no ligand was added. Values above the bars represent the fold stimulation by BMP2. B) LβT2 cells seeded in 24-well plates were transfected with 100 ng/well of the indicated constitutively active type I receptor constructs and murine -568/+15 *Id3*-luc. Cells were starved in serum-free medium for 24 h prior to analysis. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which the empty vector was transfected. C) Pituitary cultures from transgenic *Bmpr1a*$^{flox/flox}$

mice infected with adenovirus expressing GFP or Cre recombinase and GFP were pre-treated with 10 μM SB431542 and then treated with 25 ng/ml activin A or 50 ng/ml BMP2. Changes in *Id3* mRNA expression were measured by qRT-PCR. Data are presented relative to untreated cultures infected with GFP-expressing virus. Treatments were performed in triplicate. The figure shown is from a representative experiment. Results of the replicate experiments are presented in Supplemental Fig.S2. D) LβT2 cells seeded in 24-well plates were transfected with murine -568/+15 *Id3*-luc and 5 nM of the control siRNA or siRNAs for the indicated BMP type II receptors and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no siRNAs or ligands were included. Values above the bars represent the fold stimulation by BMP2.

Figure 4.4. A) LβT2 cells seeded in 24-well plates were transfected with murine -568/+15 Id3-luc and 5 nM of the indicated siRNAs and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no siRNAs or ligand were included. Values above the bars represent the fold stimulation by BMP2. B) LβT2 cells seeded in 24-well plates were transfected with 100 ng/well of the indicated SMAD expression constructs and murine -568/+15 *Id3*-luc. Cells were treated with 25 ng/ml BMP2 in serum-free medium for 24 h. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group (pcDNA3, no ligand). BMP2 treated and untreated groups were analyzed separately.

Figure 4.5. A) LβT2 cells seeded in 24-well plates were transfected with different lengths of murine *Id3* promoter-reporters and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with the empty vector, pGL3-Basic. Values beside the bars represent the fold stimulation by BMP2 for each reporter. B) EMSAs were performed with the indicated radio-labeled (*) probes corresponding to -568/-502 of the murine *Id3* promoter. Nuclear extracts were prepared from LβT2 cells treated with 10 μM SB431542 in serum-free medium. In competition experiments, unlabeled probes were included at 100X higher concentration than the radio-labeled probes. The figure shown is representative of three experiments. Free probes are not pictured. C) EMSAs were performed as in panel B with the -528/-502 radio-labeled probe. Here, competitions were performed with 3-bp scanning mutants of the -528/-

502 probe (Mut1-9, shown at top). The schematic at the top reflects the relative positions of the nucleotides mediating binding of complexes A through D. D) Mut2 through Mut6 defined in panel C were introduced into the -568/+15 *Id3* reporter and the constructs transfected into LβT2 cells seeded in 24-well plates. Cells were treated with BMP2 as described in panel A. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL3-Basic. Values beside the bars represent the fold stimulation by BMP2 for each reporter. E) LβT2 cells seeded in 24-well plates were transfected with wild-type or Mut4 -568/+15 *Id3*-luc and 100 ng/well of each SMAD5 and SMAD4. Cells were then treated with 25 ng/ml BMP2 in serum-free medium containing 10 μM SB431542 for 24 h. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with the wild-type reporter and pcDNA3.

Figure 4.6. A) LβT2 cells seeded in 24-well plates were transfected with different lengths of human *ID3* promoter-reporters and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with the empty vector, pGL2-Basic. B) The equivalents of Mut4 and Mut5 (each represented as an X on the promoter) in the murine *Id3* promoter were introduced into the human -653/+402 *ID3* promoter-reporter and the constructs transfected into LβT2 cells seeded in 24-well plates. Cells were then treated as in panel A. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL2-Basic.

Figure 4.7. A) LβT2 cells seeded in 24-well plates were transfected with varying lengths of murine *Id3* promoter-reporters and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of nine independent experiments and are presented relative to the untreated cells transfected with the empty vector, pGL3-Basic. B) Mutations were introduced to the distal or proximal BRE in the murine -3740/+24 *Id3*-luc alone or together, and the constructs transfected into LβT2 cells seeded in 24-well plates. The X in the dBRE represents the mutation TGGCGCC→ TGGTGCT, whereas the X in pBRE represents Mut4 (left X) or Mut5 (right X) as described in Figure 5C. Cells were then treated as in panel A. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL3-Basic.

C) NIH3T3 cells seeded in 24-well plates were transfected and treated as in panel A. Treatments were performed in triplicate. The data reflect the mean (+SEM) of four independent experiments and are presented relative to untreated cells transfected with pGL3-Basic. D) NIH3T3 cells seeded in 24-well plates were transfected and treated as in panel B. Treatments were performed in duplicate. The data reflect the mean (+SEM) of four independent experiments and are presented relative to untreated cells transfected with pGL3-Basic.

Supplemental Figure Legends

Figure S4.1. Panels A and B represent replicates of the experiment presented in text Figure 4.1.

Figure S4.2. Panels A and B represent replicates of the experiment presented in text Figure 4.3C.

Figure S4.3. A) Primary pituitary cultures from transgenic *Bmpr1a*$^{flox/flox}$ mice infected with GFP or Cre- GFP recombinase expressing adenovirus. Representative images of cells (10X magnification) under brightfield and fluorescence conditions. B) PCR was performed on genomic DNA extracted from control cultures infected with adenovirus expressing GFP or GFP/Cre recombinase. Fx2 and Fx4 primers were designed to detect the intact floxed *Bmpr1a* allele, with an expected PCR product of 230 bp. Fx1 and Fx4 primers were designed to detect the recombined *Bmpr1a* allele, with an expected PCR product of 180 bp. The figure shown is a representative of three experiments, all of which produced comparable results. C) The cDNA used in text Figure 4.3C was used to detect changes in *Bmpr1a* expression by qRT-PCR. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which the cultures were infected with GFP alone and no ligand was included.

Figure S4.4. A) CHO cells seeded in 6-well plates were transfected with FLAG-human SMAD1 (SMAD1-resistant) or the SMAD1 construct modified to contain sequence targeted by the murine *Smad1* siRNA1 or 2 (SMAD1-sensitive1 and SMAD1-sensitive2, respectively). Cells were co-transfected with no siRNA, 10 nM control siRNA, or one of the two *Smad1* siRNAs. Whole cell protein lysates were collected after 24 h and subjected to western blot analyses. Antibodies for FLAG were used to detect SMAD1 expression; β-actin was used as the loading control. B) CHO cells seeded in 6-well plates were transfected with FLAG- murine SMAD5 or the FLAG-rat SMAD5 expression constructs together with no siRNA, 10 nM control, or murine *Smad5* siRNA. Whole cell protein lysates were collected and subjected to western blot analyses as in panel A. C) CHO cells were transfected as in panel A with rat FLAG-SMAD8 (SMAD8-resistant) or the SMAD8 construct modified to contain the sequence targeted by the murine *Smad8* siRNA1 or 2 (SMAD8-sensitive1 and SMAD8-sensitive2 respectively). Whole cell protein lysates were collected and subjected to western blot analyses as in panel A.

Figure S4.5. LβT2 cells seeded in 24-well plates were transfected with the murine -568/+15 *Id3*-luc, 5 nM *Smad5* siRNA, and 100 ng/well rat SMAD5 expression construct (resistant to *Smad5* siRNA).

The cells were then treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group (pcDNA3, no siRNA). Values above the bars represent the fold stimulation by BMP2; values with different symbols were statistically different, whereas those sharing symbols did not differ.

Figure S4.6. A) EMSAs were performed as in text Figure 4.5C with the -568/-542 radio-labeled probe. Competitions were performed with 3-bp scanning mutants of the -568/-542 probe (Mut1-9, shown at top). The schematic at the top reflects the relative positions of the nucleotides mediating binding of complexes A and B. B) Mut2 through Mut5 defined in panel A were introduced into the -568/+15 *Id3* reporter and the constructs transfected into LβT2 cells seeded in 24-well plates. Cells were treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL3-Basic.

Figure S4.7. A) LβT2 cells seeded in 10-cm dishes were treated with 50 ng/ml BMP2 for 1h. Chromatin was subjected to ChIP analysis using SMAD1 Ab or a control mouse IgG. DNA obtained from ChIP was analyzed by qRT-PCR. Primers were designed against the distal or proximal BREs, as well as a non-specific (NS) region located between the two response elements. Data normalized against input fraction and control mouse IgG ($2^{-\Delta\Delta Ct}$) are plotted as fold enrichment. The data reflect the mean (+SEM) of three independent experiments. B) CHO cells seeded in 10-cm dishes were transfected with 4 μg of HA-GATA4, 4 μg of FLAG-SMAD1, or both expression vectors together. Cells were treated overnight with 25 ng/ml BMP2 before whole cell lysates were collected for HA-immunoprecipitation (IP) analysis. Immunoprecipitated proteins were subjected to immunoblot (IB) analysis with FLAG and HA antibodies.

Figure S4.8. Sequence alignment of proximal and distal *ID3/Id3* promoters in human (NM_002167.3, NC_000001.10), mouse (NM_008321.2, NC_000070.5), rat (NM_013058.2, NC_005104.2), cow (NM_001014950.1, NC_007300.4), chimpanzee (XM_001165695.1,NC_006468.2), and dog (NM_001003025.2 ,NC_006584.2). Defined *cis*-elements are labeled and boxed. Nucleotides are numbered relative to the transcription start site. Nucleotides not conserved across species are marked with an asterisk (*). Accession numbers provided above correspond to mRNA and genomic sequences, respectively. mRNA sequences were used to define the start of transcription.

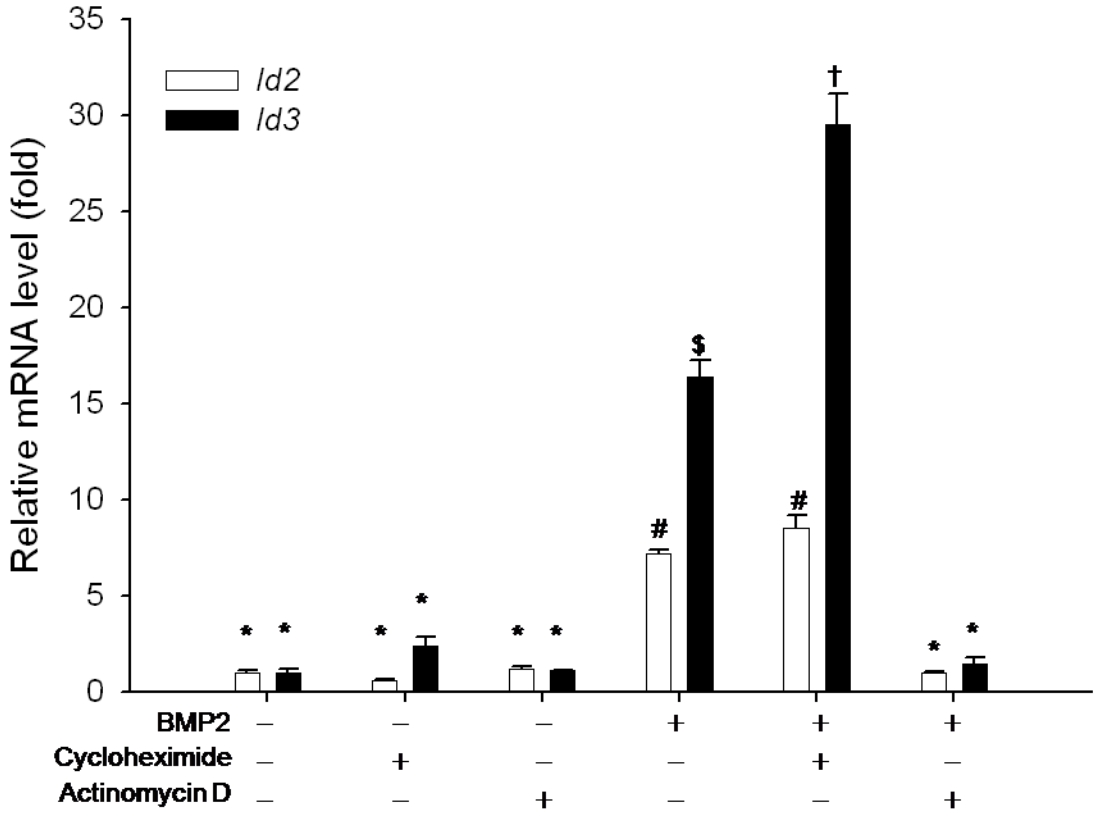

Id2
Id3
Relative mRNA level (fold)
35
30
25
20
15
10
5
0
BMP2
Cycloheximide
Actinomycin D
*
#
$
†

Figure 4.1

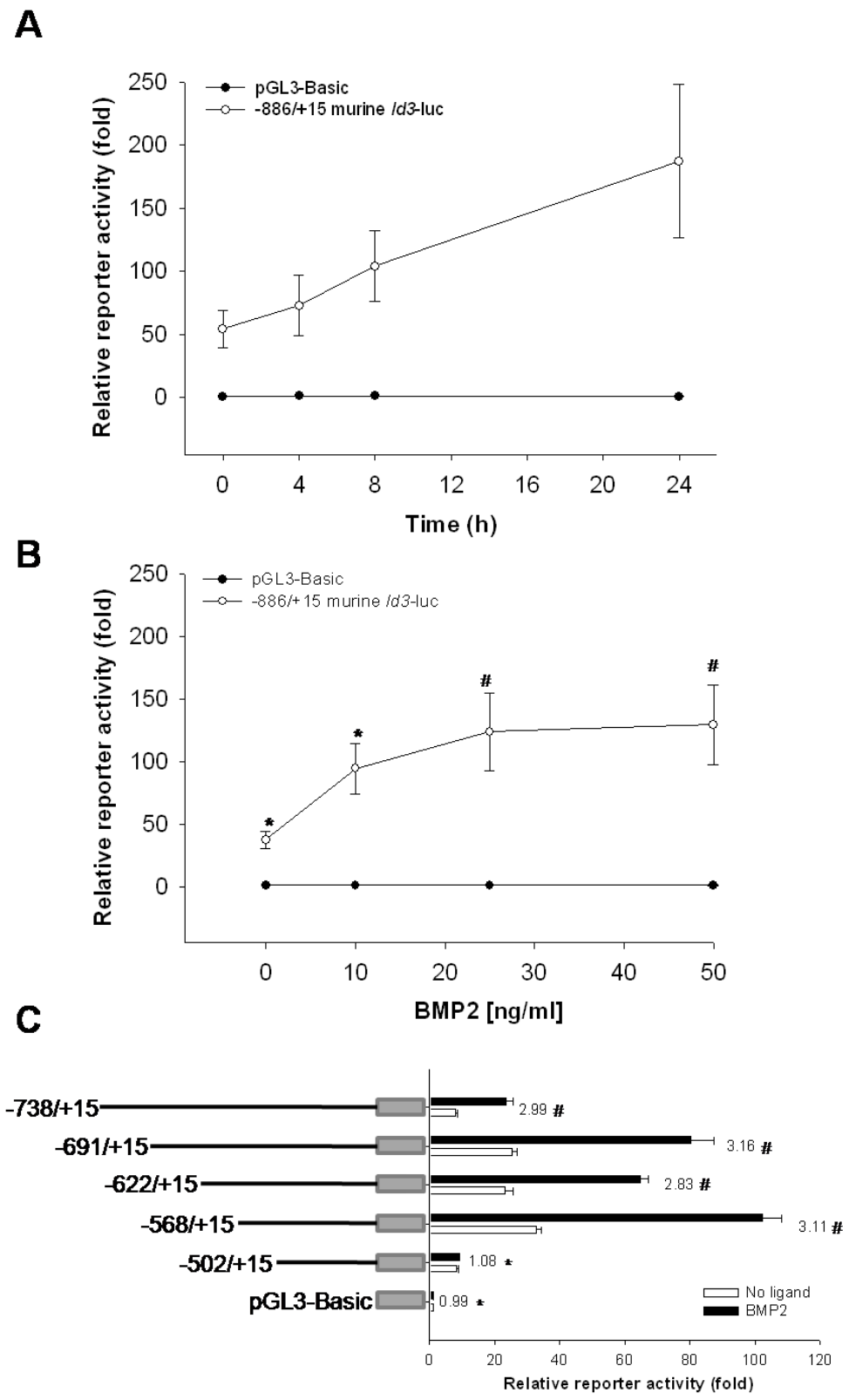

A
pGL3-Basic
-886/+15 murine Id3-luc
Relative reporter activity (fold)
Time (h)
B
pGL3-Basic
-886/+15 murine Id3-luc
Relative reporter activity (fold)
BMP2 [ng/ml]
C
-738/+15
-691/+15
-622/+15
-568/+15
-502/+15
pGL3-Basic
2.99 #
3.16 #
2.83 #
3.11 #
1.08 *
0.99 *
No ligand
BMP2
Relative reporter activity (fold)

Figure 4.2

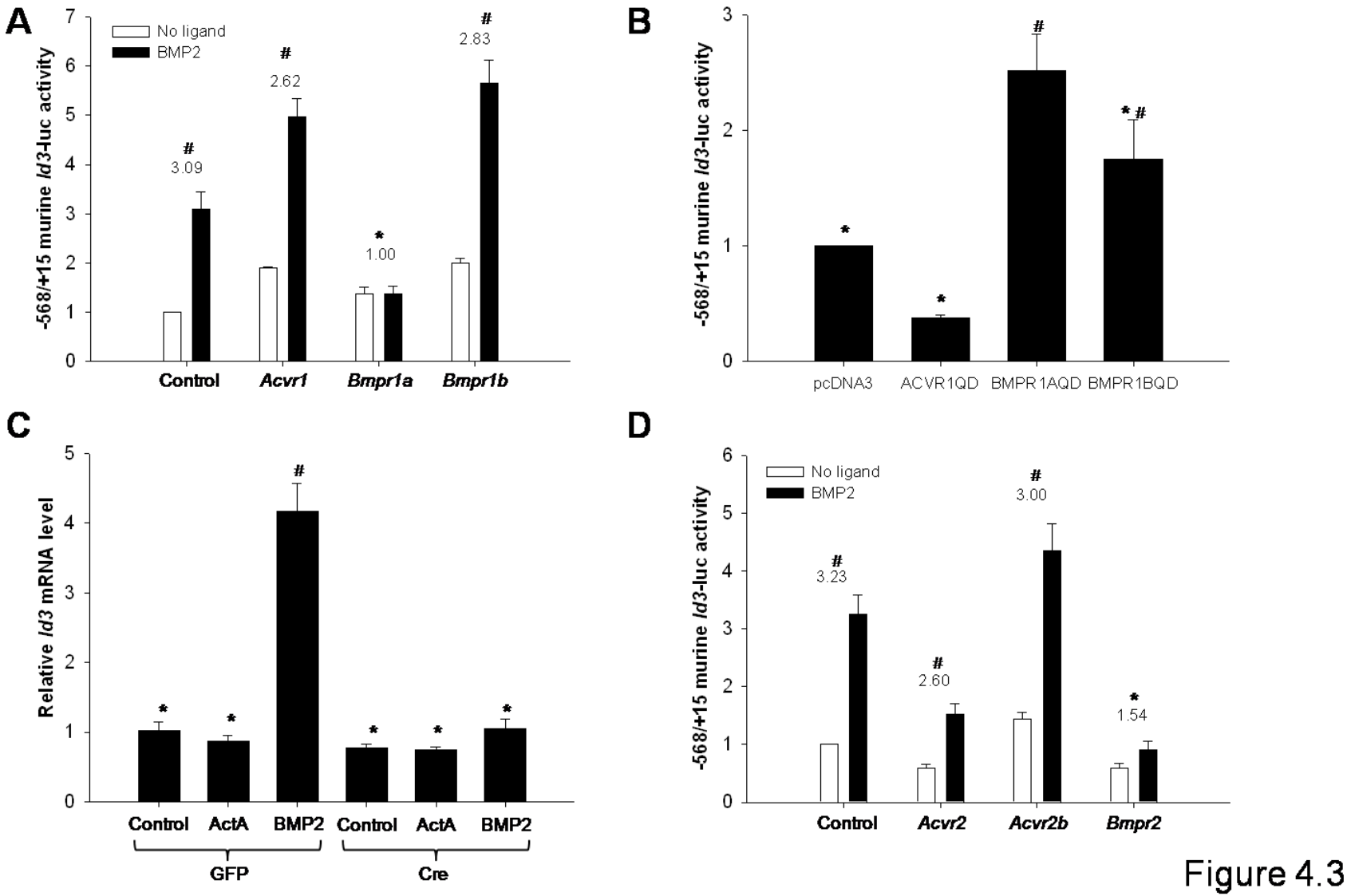

A
No ligand
BMP2
-568/+15 murine Id3-luc activity
3.09
2.62
1.00
2.83
Control
Acvr1
Bmpr1a
Bmpr1b
B
-568/+15 murine Id3-luc activity
pcDNA3
ACVR1QD
BMPR1AQD
BMPR1BQD
C
Relative Id3 mRNA level
Control
ActA
BMP2
Control
ActA
BMP2
GFP
Cre
D
No ligand
BMP2
-568/+15 murine Id3-luc activity
3.23
2.60
3.00
1.54
Control
Acvr2
Acvr2b
Bmpr2

Figure 4.3

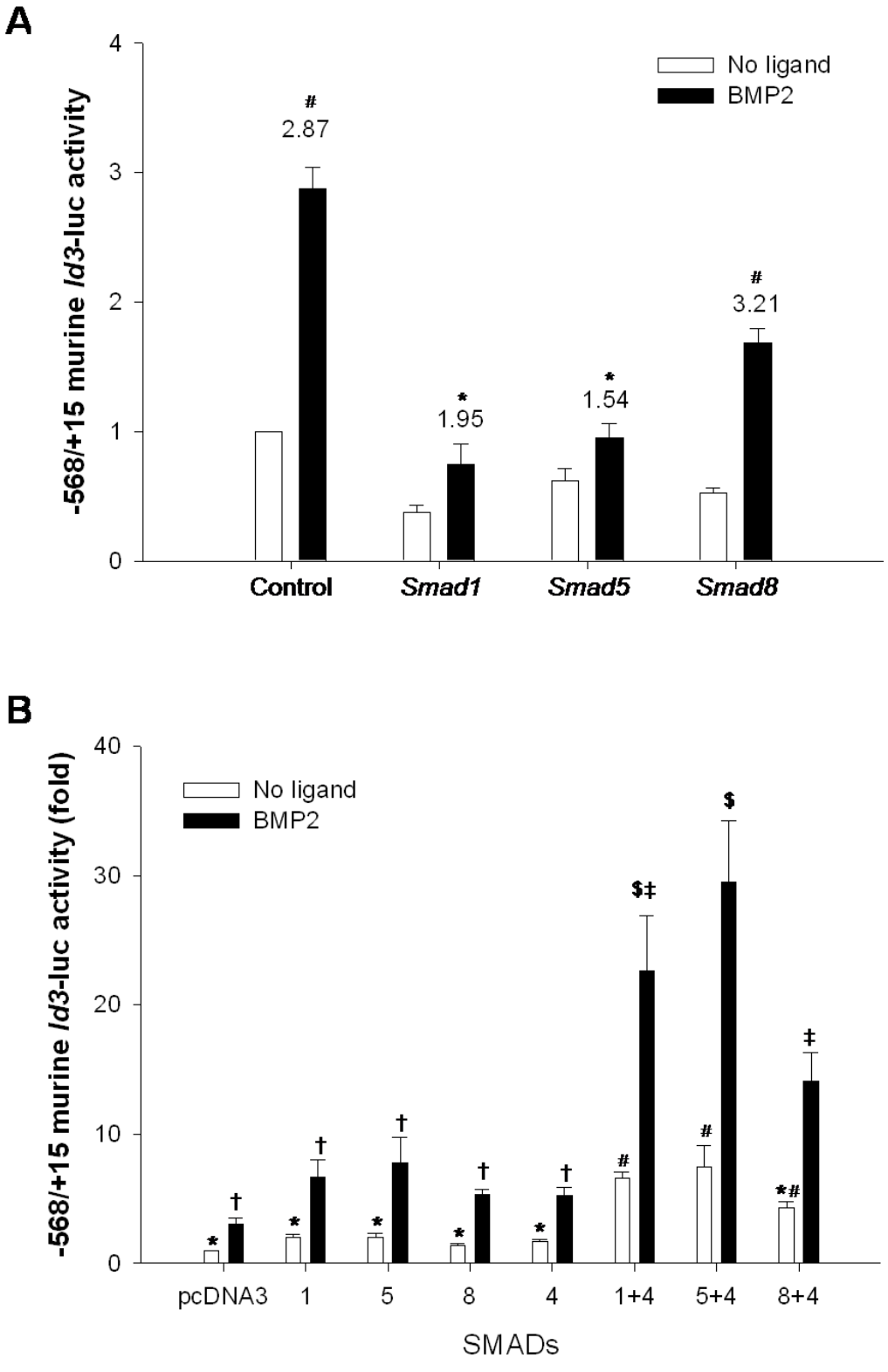

A
-568/+15 murine Id3-luc activity
No ligand
BMP2
#
2.87
*
1.95
*
1.54
#
3.21
Control
Smad1
Smad5
Smad8
B
-568/+15 murine Id3-luc activity (fold)
No ligand
BMP2
pcDNA3
1
5
8
4
1+4
5+4
8+4
SMADs

Figure 4.4

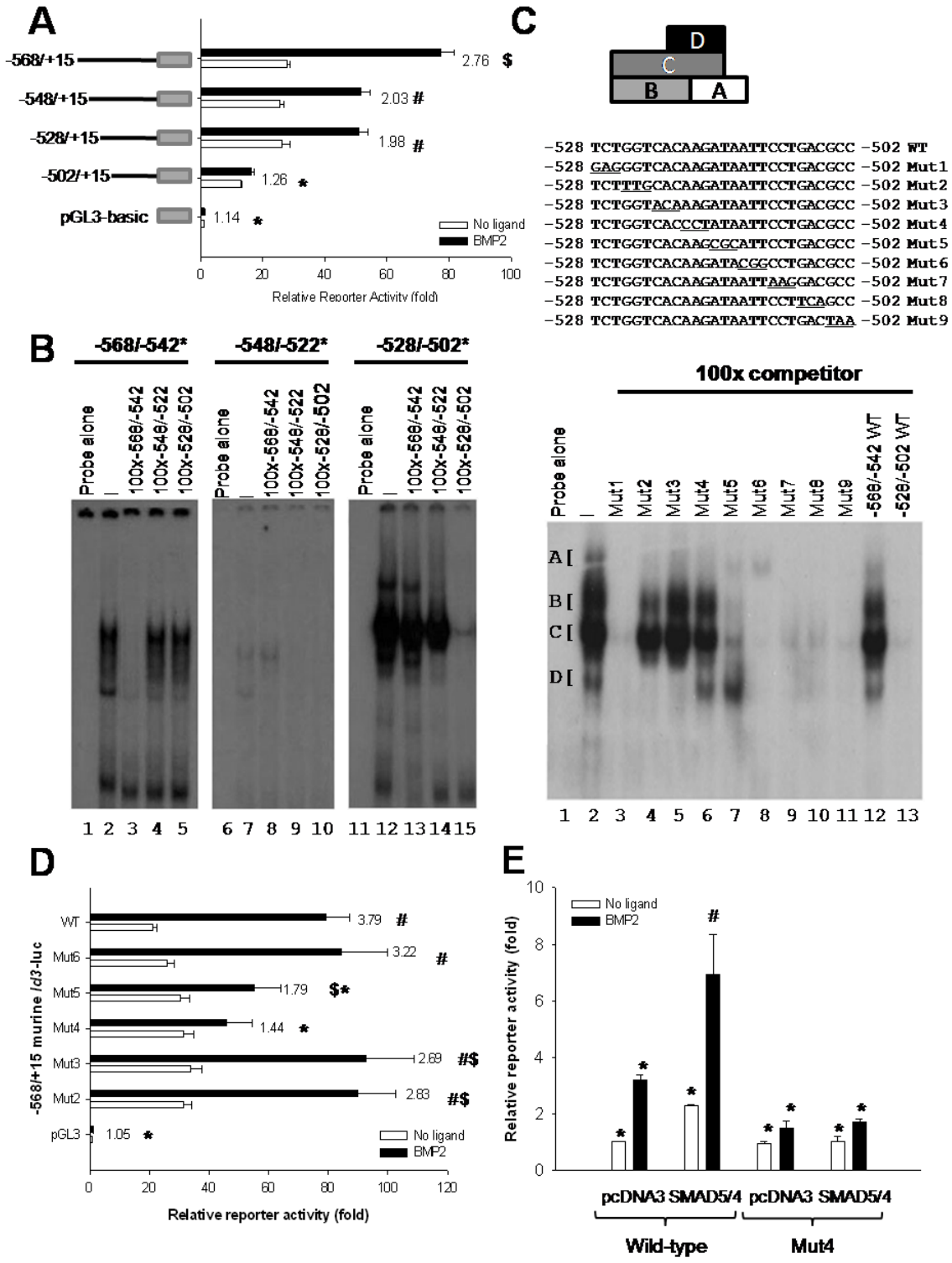

A
-568/+15
-548/+15
-528/+15
-502/+15
pGL3-basic
2.76 $
2.03 #
1.98 #
1.26 *
1.14 *
No ligand
BMP2
0 20 40 60 80 100
Relative Reporter Activity (fold)
C
D
C
B
A
-528 TCTGGTCACAAGATAATTCCTGACGCC -502 WT
-528 GAGGGTCACAAGATAATTCCTGACGCC -502 Mut1
-528 TCTTTGCACAAGATAATTCCTGACGCC -502 Mut2
-528 TCTGGTACAAAGATAATTCCTGACGCC -502 Mut3
-528 TCTGGTCACCCTATAATTCCTGACGCC -502 Mut4
-528 TCTGGTCACAAGCGCATTCCTGACGCC -502 Mut5
-528 TCTGGTCACAAGATACGGCCTGACGCC -502 Mut6
-528 TCTGGTCACAAGATAATTAAGGACGCC -502 Mut7
-528 TCTGGTCACAAGATAATTCCTTCAGCC -502 Mut8
-528 TCTGGTCACAAGATAATTCCTGACTAA -502 Mut9
B
-568/-542*
-548/-522*
-528/-502*
Probe alone
—
100x-568/-542
100x-548/-522
100x-528/-502
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
100x competitor
Probe alone
—
Mut1
Mut2
Mut3
Mut4
Mut5
Mut6
Mut7
Mut8
Mut9
-568/-542 WT
-528/-502 WT
A [
B [
C [
D [
1 2 3 4 5 6 7 8 9 10 11 12 13
D
-568/+15 murine /d3-luc
WT
Mut6
Mut5
Mut4
Mut3
Mut2
pGL3
3.79 #
3.22 #
1.79 $*
1.44 *
2.69 #$
2.83 #$
1.05 *
No ligand
BMP2
0 20 40 60 80 100 120
Relative reporter activity (fold)
E
Relative reporter activity (fold)
No ligand
BMP2
0 2 4 6 8 10
pcDNA3 SMAD5/4 pcDNA3 SMAD5/4
Wild-type
Mut4

Figure 4.5

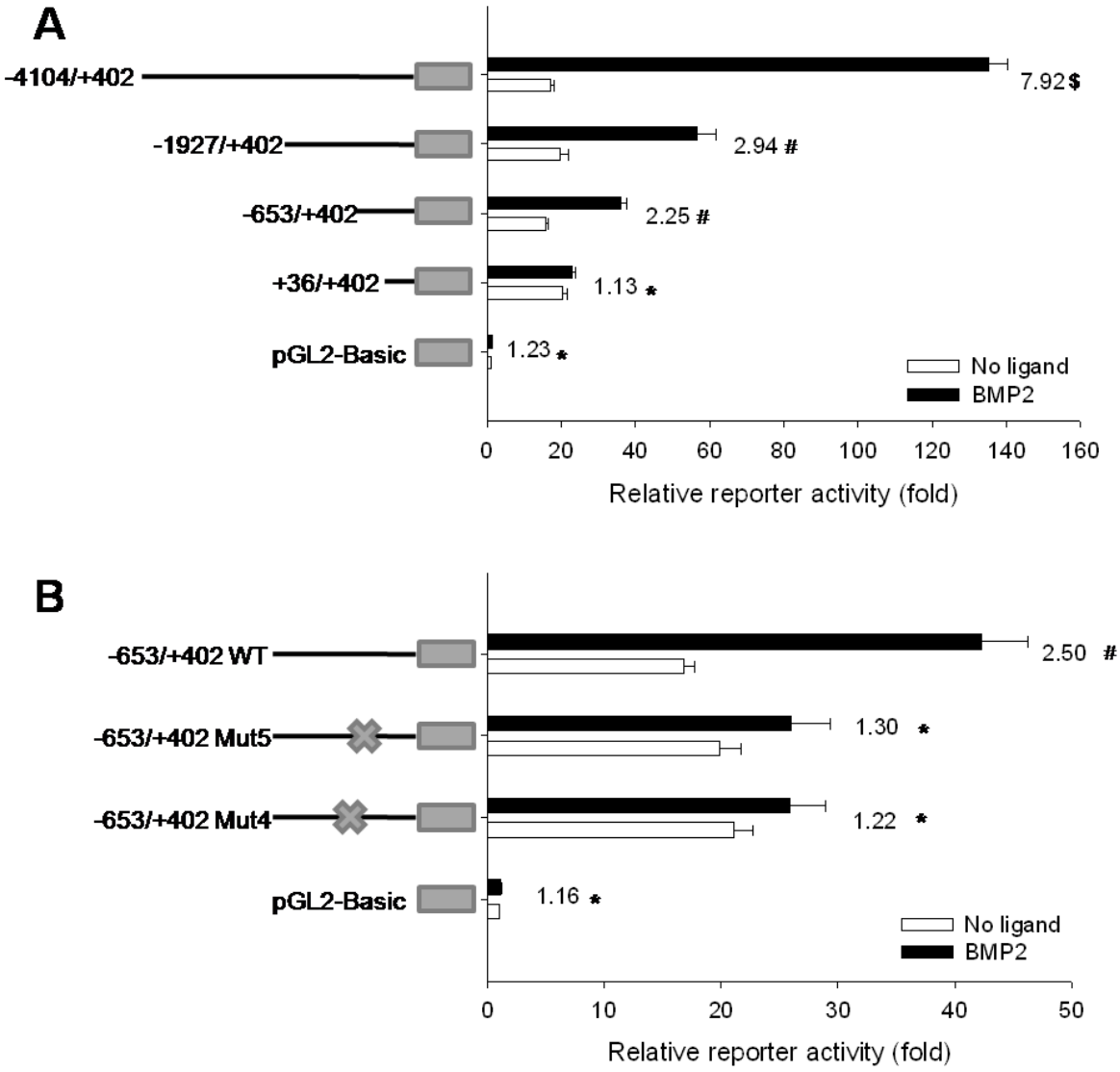

A
-4104/+402
-1927/+402
-653/+402
+36/+402
pGL2-Basic
7.92 $
2.94 #
2.25 #
1.13 *
1.23 *
No ligand
BMP2
0
20
40
60
80
100
120
140
160
Relative reporter activity (fold)
B
-653/+402 WT
-653/+402 Mut5
-653/+402 Mut4
pGL2-Basic
2.50 #
1.30 *
1.22 *
1.16 *
No ligand
BMP2
0
10
20
30
40
50
Relative reporter activity (fold)

Figure 4.6

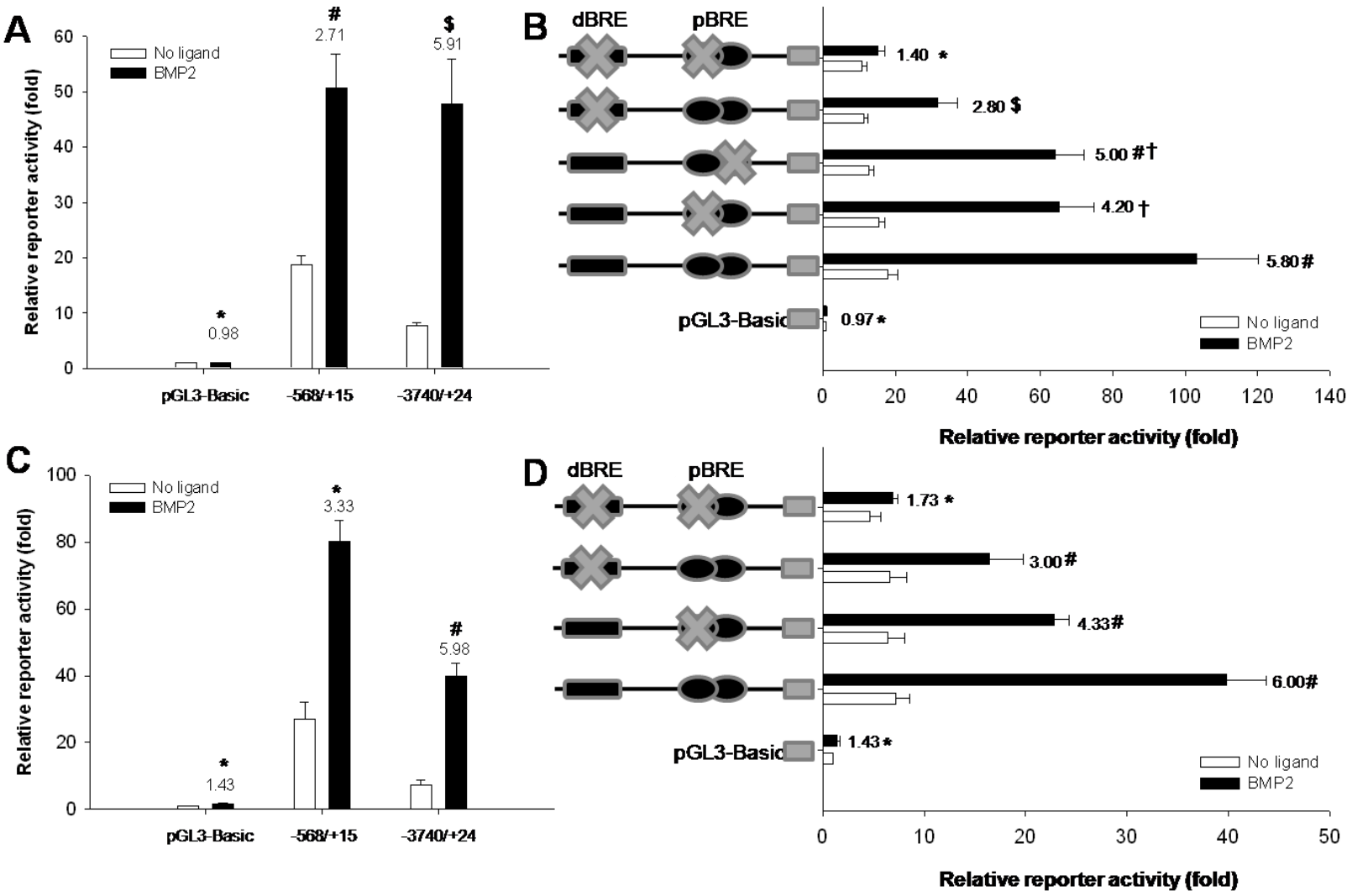

A
Relative reporter activity (fold)
No ligand
BMP2
0.98
2.71
5.91
pGL3-Basic
-568/+15
-3740/+24
B
dBRE
pBRE
1.40 *
2.80 $
5.00 #†
4.20 †
5.80 #
pGL3-Basic
0.97 *
No ligand
BMP2
Relative reporter activity (fold)
C
Relative reporter activity (fold)
No ligand
BMP2
1.43
3.33
5.98
pGL3-Basic
-568/+15
-3740/+24
D
dBRE
pBRE
1.73 *
3.00 #
4.33 #
6.00 #
pGL3-Basic
1.43 *
No ligand
BMP2
Relative reporter activity (fold)

Figure 4.7

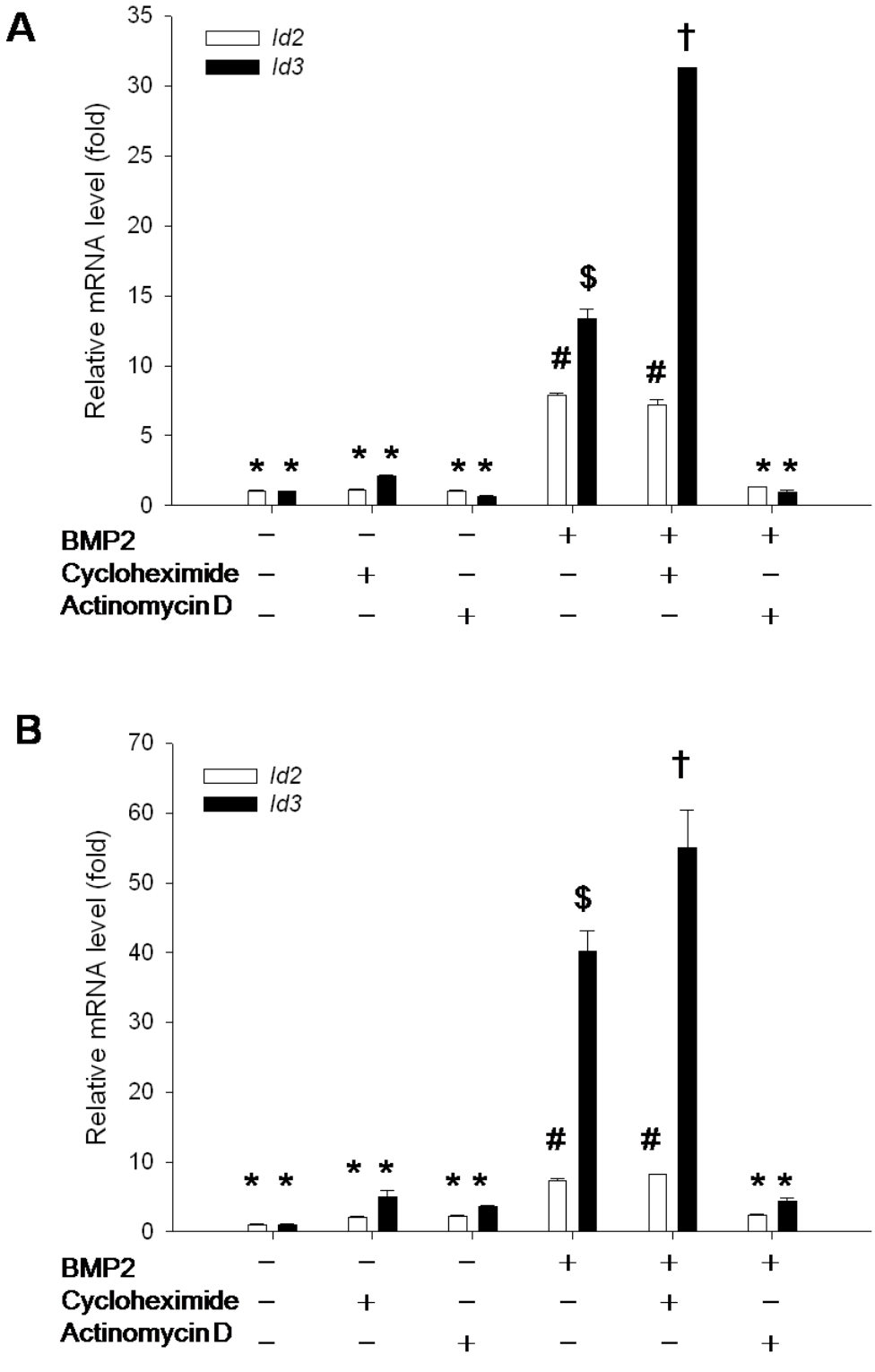

A
Id2
Id3
Relative mRNA level (fold)
35
30
25
20
15
10
5
0
†
$
#
#
* *
* *
* *
* *
BMP2 – – – + + +
Cycloheximide – + – – + –
Actinomycin D – – + – – +
B
Id2
Id3
Relative mRNA level (fold)
70
60
50
40
30
20
10
0
†
$
#
#
* *
* *
* *
* *
BMP2 – – – + + +
Cycloheximide – + – – + –
Actinomycin D – – + – – +

Figure S4.1

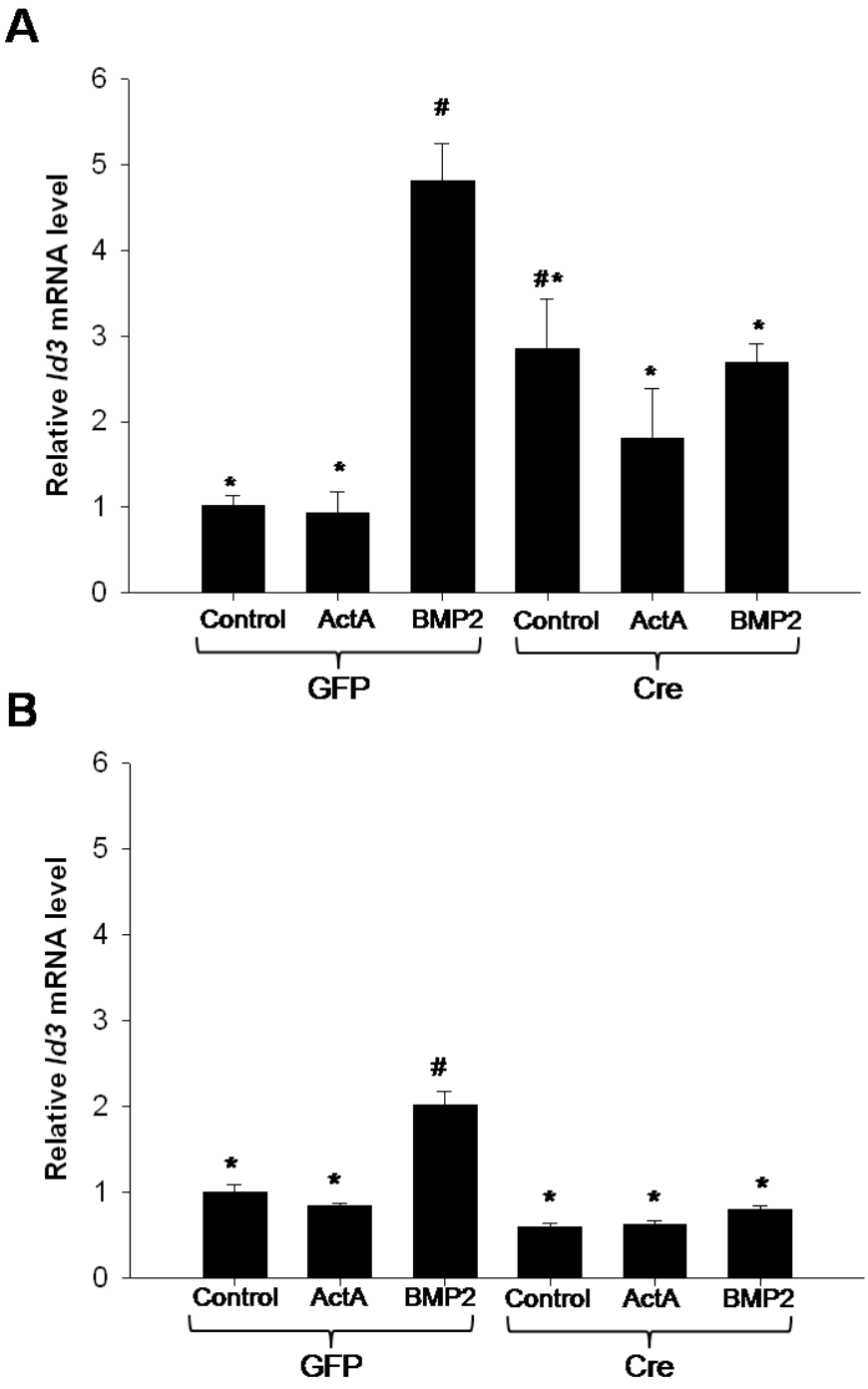

A
Relative Id3 mRNA level
0
1
2
3
4
5
6
#
#*
*
Control
ActA
BMP2
Control
ActA
BMP2
GFP
Cre
B
Relative Id3 mRNA level
0
1
2
3
4
5
6
#
*
Control
ActA
BMP2
Control
ActA
BMP2
GFP
Cre

Figure S4.2

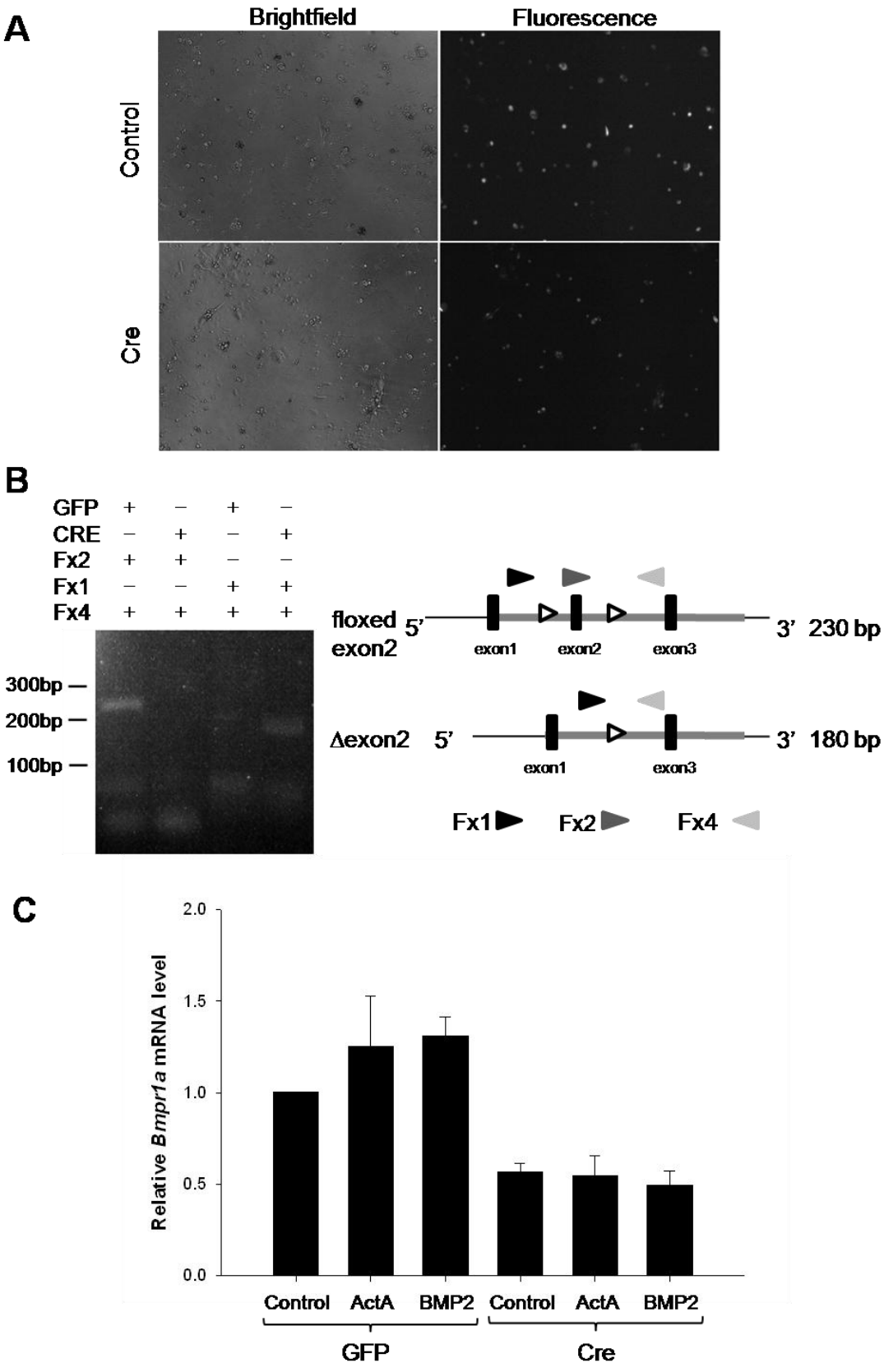

A
Brightfield
Fluorescence
Control
Cre
B
GFP + − + −
CRE − + − +
Fx2 + + − −
Fx1 − − + +
Fx4 + + + +
300bp
200bp
100bp
floxed exon2
5'
3' 230 bp
exon1
exon2
exon3
Δexon2
5'
3' 180 bp
exon1
exon3
Fx1
Fx2
Fx4
C
Relative Bmpr1a mRNA level
2.0
1.5
1.0
0.5
0.0
Control
ActA
BMP2
Control
ActA
BMP2
GFP
Cre

Figure S4.3

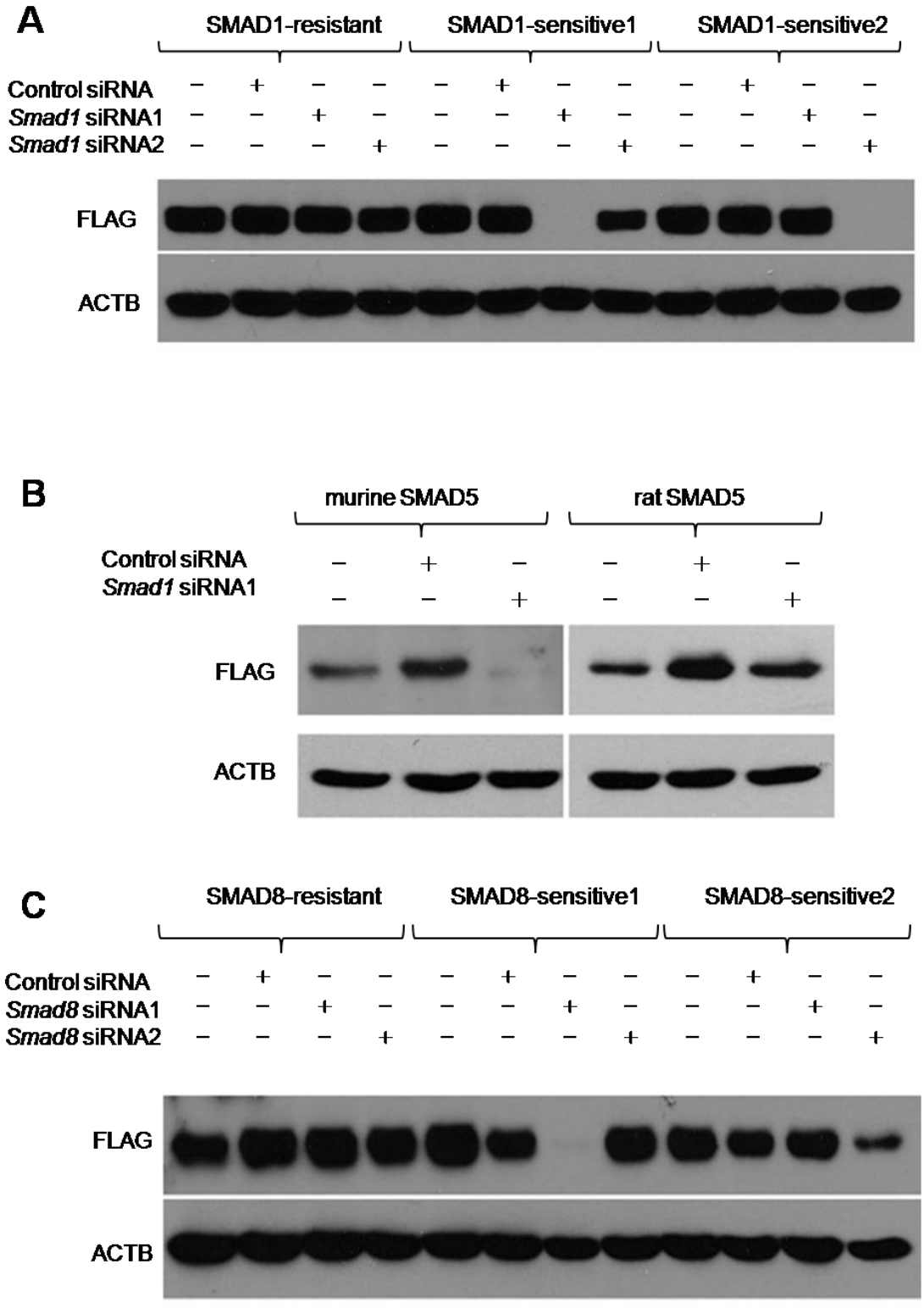

A
SMAD1-resistant
SMAD1-sensitive1
SMAD1-sensitive2
Control siRNA
Smad1 siRNA1
Smad1 siRNA2
FLAG
ACTB
B
murine SMAD5
rat SMAD5
Control siRNA
Smad1 siRNA1
FLAG
ACTB
C
SMAD8-resistant
SMAD8-sensitive1
SMAD8-sensitive2
Control siRNA
Smad8 siRNA1
Smad8 siRNA2
FLAG
ACTB

Figure S4.4

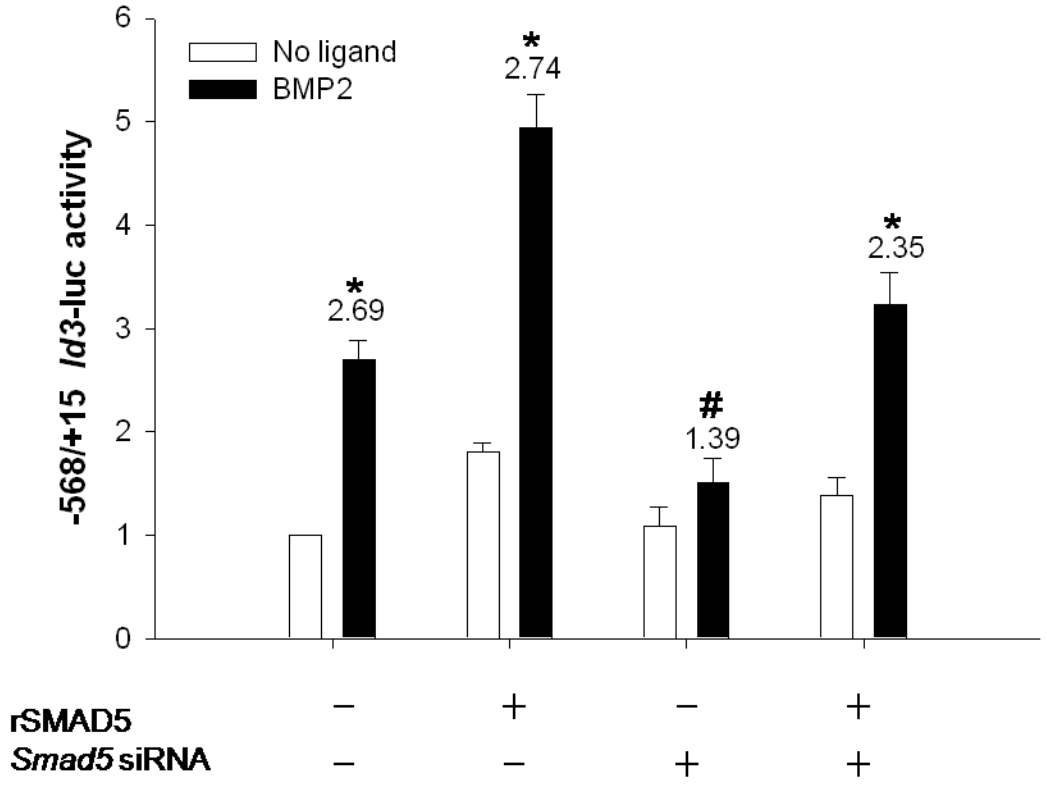
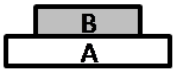
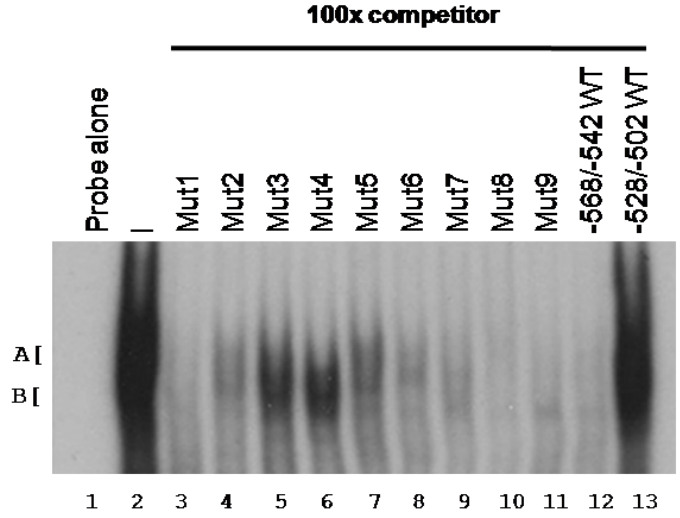

6
5
4
3
2
1
0
-568/+15 Id3-luc activity
No ligand
BMP2
*
2.74
*
2.69
#
1.39
*
2.35
rSMAD5
Smad5 siRNA
−
+
−
+
−
−
+
+

Figure S4.5

A



−568CATTGTAACCTCAGCTTCACCGCAATT−542 WT
−568ACGTGTAACCTCAGCTTCACCGCAATT−542 Mut1
−568CATGTGAACCTCAGCTTCACCGCAATT−542 Mut2
−568CATTGTCCACTCAGCTTCACCGCAATT−542 Mut3
−568CATTGTAACAGAAGCTTCACCGCAATT−542 Mut4
−568CATTGTAACCTCCTATTCACCGCAATT−542 Mut5
−568CATTGTAACCTCAGCGGAACCGCAATT−542 Mut6
−568CATTGTAACCTCAGCTTCCAAGCAATT−542 Mut7
−568CATTGTAACCTCAGCTTCACCTACATT−542 Mut8
−568CATTGTAACCTCAGCTTCACCGCACGG−542 Mut9



B

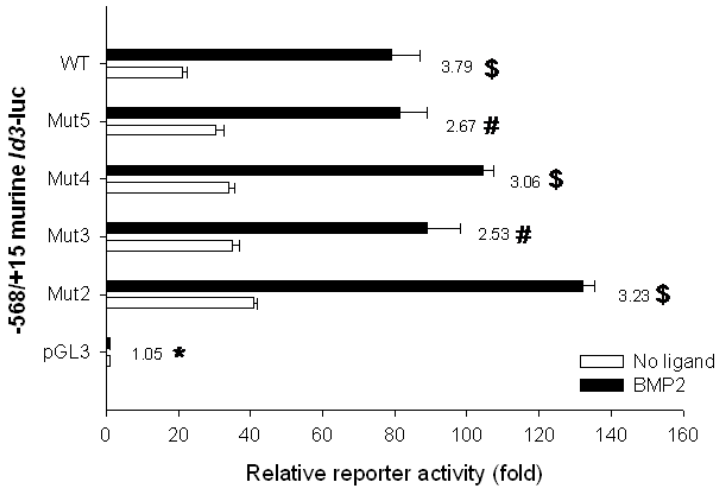
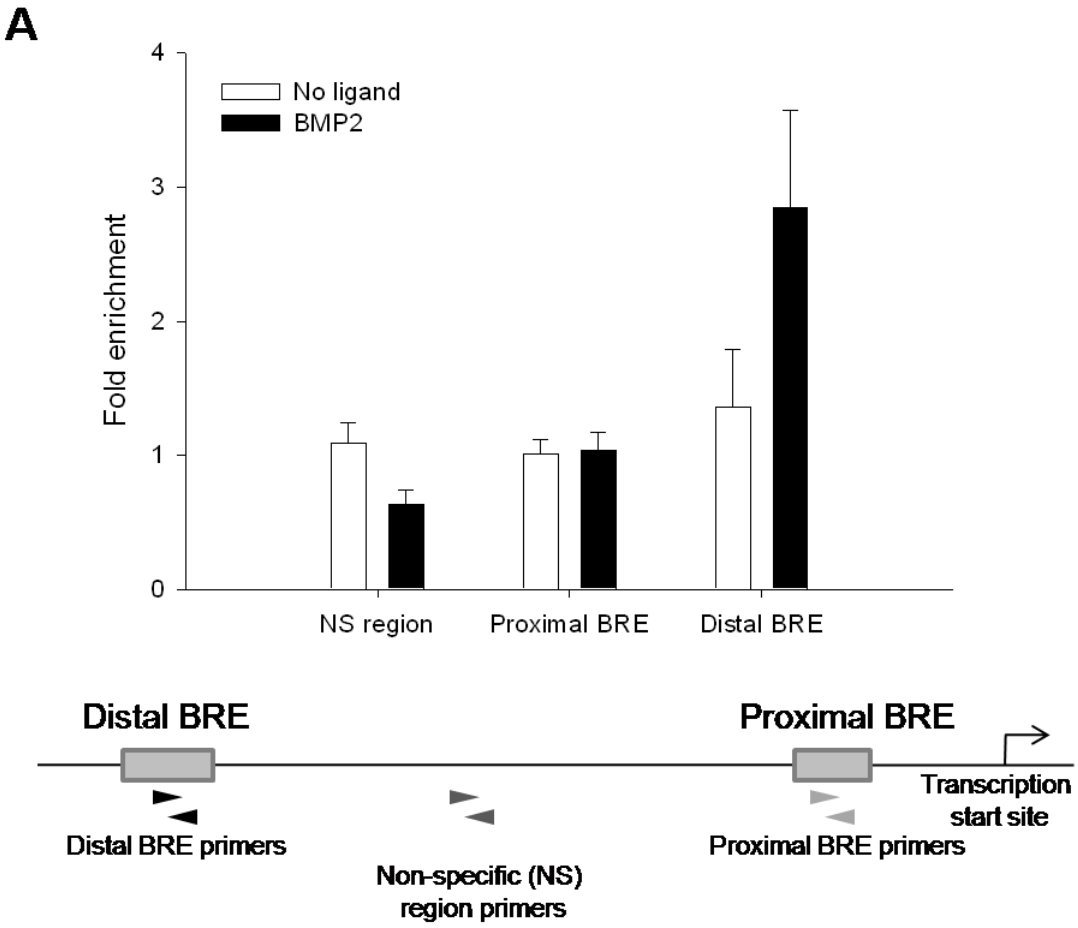


Figure S4.6


A
4
3
2
1
0
Fold enrichment
No ligand
BMP2
NS region
Proximal BRE
Distal BRE
Distal BRE
Proximal BRE
Transcription start site
Distal BRE primers
Non-specific (NS) region primers
Proximal BRE primers

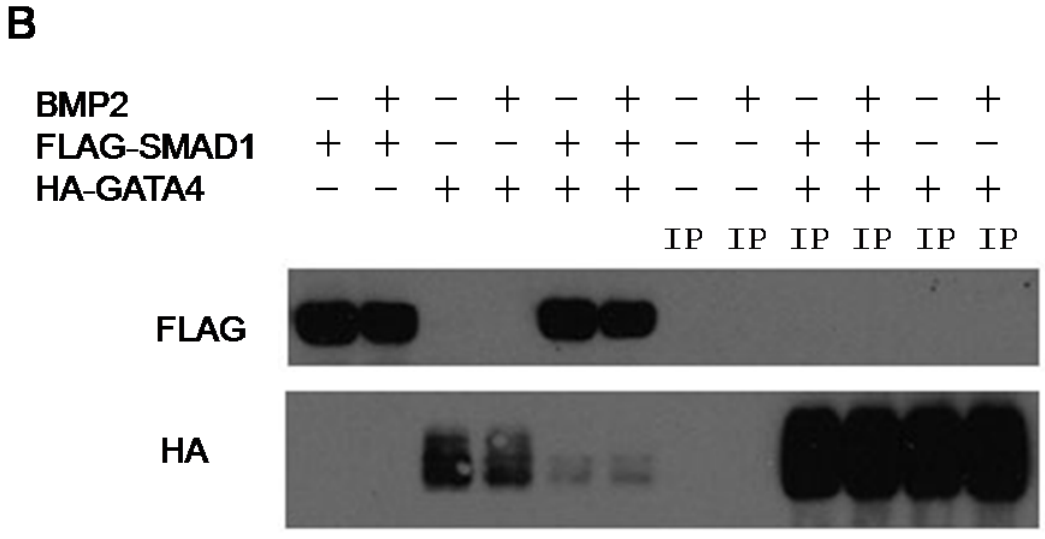

B
BMP2 − + − + − + − + − + − +
FLAG-SMAD1 + + − − + + − − + + − −
HA-GATA4 − − + + + + − − + + + +
IP IP IP IP IP IP
FLAG
HA

Figure S4.7

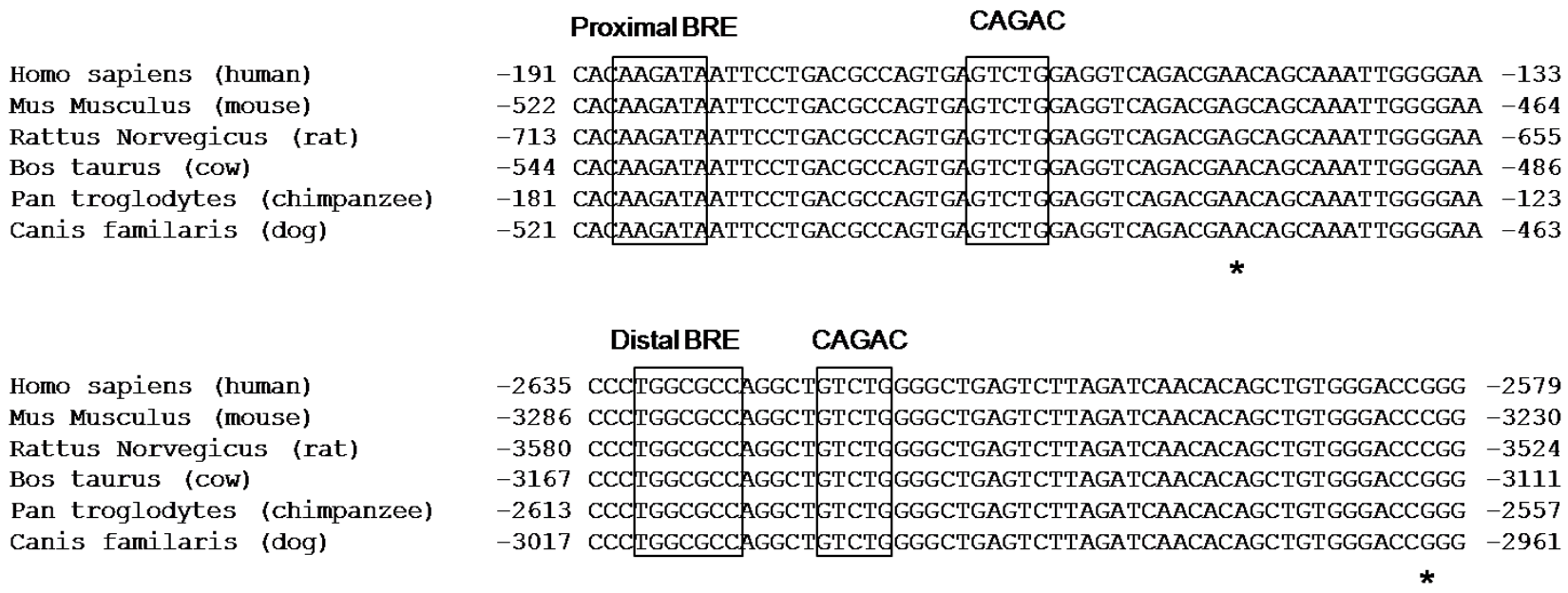

Proximal BRE
CAGAC
Homo sapiens (human) -191 CACAAGATAATTCCTGACGCCAGTGAGTCTGGAGGTCAGACGAACAGCAAATTGGGGAA -133
Mus Musculus (mouse) -522 CACAAGATAATTCCTGACGCCAGTGAGTCTGGAGGTCAGACGAGCAGCAAATTGGGGAA -464
Rattus Norvegicus (rat) -713 CACAAGATAATTCCTGACGCCAGTGAGTCTGGAGGTCAGACGAGCAGCAAATTGGGGAA -655
Bos taurus (cow) -544 CACAAGATAATTCCTGACGCCAGTGAGTCTGGAGGTCAGACGAACAGCAAATTGGGGAA -486
Pan troglodytes (chimpanzee) -181 CACAAGATAATTCCTGACGCCAGTGAGTCTGGAGGTCAGACGAACAGCAAATTGGGGAA -123
Canis familaris (dog) -521 CACAAGATAATTCCTGACGCCAGTGAGTCTGGAGGTCAGACGAACAGCAAATTGGGGAA -463
*
Distal BRE
CAGAC
Homo sapiens (human) -2635 CCCTGGCGCCAGGCTGTCTGGGGCTGAGTCTTAGATCAACACAGCTGTGGGACCGGG -2579
Mus Musculus (mouse) -3286 CCCTGGCGCCAGGCTGTCTGGGGCTGAGTCTTAGATCAACACAGCTGTGGGACCCGG -3230
Rattus Norvegicus (rat) -3580 CCCTGGCGCCAGGCTGTCTGGGGCTGAGTCTTAGATCAACACAGCTGTGGGACCCGG -3524
Bos taurus (cow) -3167 CCCTGGCGCCAGGCTGTCTGGGGCTGAGTCTTAGATCAACACAGCTGTGGGACCGGG -3111
Pan troglodytes (chimpanzee) -2613 CCCTGGCGCCAGGCTGTCTGGGGCTGAGTCTTAGATCAACACAGCTGTGGGACCGGG -2557
Canis familaris (dog) -3017 CCCTGGCGCCAGGCTGTCTGGGGCTGAGTCTTAGATCAACACAGCTGTGGGACCGGG -2961
*

Figure S4.8

Supplementary Table S4.1. Primers

Target	Forward Primer	Reverse Primer	Template DNA
-3740/+24 Id3-luc	GCGACGCGTATAGCTTACAGTTCTGCCAGCTC	GATCTCGAGGACACCTAAAGCAGCAAACAGTG	Mouse genomic DNA
FLAG-SMAD8	CGGAATTCCACCCCAGCACCCCCATCAGC	GCTCTAGATTAAGACACTGAAGAAATAGG	Myc-SMAD8
-738/+15 *Id3*-luc	GCGACGCGTGTTCTCGGTGGAAACGGTCCATG	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
-691/+15 *Id3*-luc	GCGACGCGTGCTGGGTCCAGACTGCTCTTA	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
-622/+15 *Id3*-luc	GCGACGCGTGTGTTCTCTGCTTAGACCTCC	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
-568/+15 *Id3*-luc	GCGACGCGTCATTGTAACCTCAGCTTCACCGC	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
-548/+15 *Id3*-luc	GCGACGCGTGCGAATTAATCTTTTCCCCCTCTGGTC	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
-528/+15 *Id3*-luc	GCGACGCGTTCTGGTCACAAGATAATTCCTGACGCC	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
-502/+15 *Id3*-luc	GCGACGCGTAGTGAGTCTGGAGGTCAGACGAG	GATCTCGAGCCCCTAGAAGTCG	-886/+15 *Id3*-luc
Rpl19	CGGGAATCCAAGAAGATTGA3	TTCAGCTTGTGGATGTGCTC3	LβT2/*Bmpr1a*$^{flox/flox}$ pituitary cultures cDNA
Id2	CTCCAAGCTCAAGGAACTGG3	ATTCAGATGCCTGCAAGGAC3	LβT2 cDNA
Id3	TTAGCCAGGTGGAAATCCTG3	TCAGTGGCAAAAGCTCCTCT3	LβT2/*Bmpr1a*$^{flox/flox}$ pituitary cultures

			cDNA
Bmpr1a	ACGCTTGCGGCCAATCGTGT	AGCTGTGAGTCTGGAGGCTGGA	*Bmpr1a*$^{flox/flox}$ pituitary cultures cDNA
Non-Specific Region	CTCCGAGACTGGCTTACCTG	CCAGATACCACGGCTTTGAT	LβT2 ChIP DNA
Proximal BRE	GCCTAGCCCAAATCTGTTTTC	ATTTGCTGCTCGTCTGACCT	LβT2 ChIP DNA
Distal BRE	ATTGGTGGGAGAGGCAGTC	GTTGAGGAATCCGCTCCTTT	LβT2 ChIP DNA
floxed exon2	Fx2: GCAGCTGCTGCTGCAGCCTCC	Fx4: TGGCTACAATTTGTCTCATGC	*Bmpr1a*$^{flox/flox}$ pituitary cultures genomic DNA
Δexon2	Fx1: GGTTTGGATCTTAACCTTAGG	Fx4: TGGCTACAATTTGTCTCATGC	*Bmpr1a*$^{flox/flox}$ pituitary cultures genomic DNA

Chapter 5: General Discussion

Activins are specific and potent stimulators of *Fshb* transcription, thus allowing for differential regulation of FSH and LH expression. We and other groups have shown that BMPs are also specific modulators of *Fshb* expression in rodents [282, 401, 405, 409]. Conversely, BMP4 inhibits *Fshb* expression in sheep [408]. Despite the difference, BMPs seem to play a *bona fide* role in regulating *Fshb* transcription. Further studies suggest that BMPs can act synergistically with activin A to stimulate *Fshb* expression in mice. BMP-mediated *Fshb* transcription is a novel area of study, and the underlying mechanisms remain to be fully determined. In my thesis, I have elucidated part of the mechanism by which BMP2 regulates *Fshb* transcription in conjunction with activin A (Figure 5.1). I first demonstrated in the LβT2 gonadotrope cell line that BMP2 acts preferentially through BMPR1A and BMPR2 to potentiate the activin A response on *Fshb* transcription, and that their loss cannot be compensated for by other BMP receptors (Chapter 2). My data suggest that the direct effects of BMP2 on FSH synthesis are modest relative to their synergistic effects with activins. This synergism appears to depend on BMP-stimulated gene expression. Using cDNA microarray analyses (Chapter 3), I identified Id proteins as BMP2 targets in LβT2 cells. This is the first time Ids were found to be expressed and regulated by BMPs in gonadotrope cells. Furthermore, Id2 and Id3 are indispensable for BMP2 to stimulate *Fshb* transcription synergistically with activin A. BMP2 stimulates *Id2* and *Id3* expression in gonadotrope cells, which act cooperatively with activins to stimulate *Fshb* expression. Indeed, Id2 and Id3 can physically interact with SMAD3, a major downstream transducer of activin signaling. However, the exact mechanism by which Id2/3 and SMAD3 act to stimulate *Fshb* transcription remains to be determined. Finally, in light of the new found role for *Id2* and *Id3* in gonadotrope cells, the last part of my thesis describes the transcriptional regulation of the *Id3* gene (Chapter 4). Similar to *Fshb*, BMP2 acts through BMPR1A and BMPR2 to stimulate *Id3* expression. I confirmed in primary pituitary cultures that *Bmpr1a* is required to mediate BMP2-induced *Id3* expression. I also identified a novel BMP2 responsive element (BRE) in the proximal *Id3* promoter. SMAD1, 5, and 4 are necessary for BMP2 to stimulate *Id3* expression; however, I could not detect their binding to the *Id3* proximal BRE. Conversely, a specific protein complex can to bind the proximal BRE of the *Id3* promoter, though its exact composition is yet to be determined. In addition, I demonstrated that this proximal BRE acts synergistically with a previously identified bipartite element in the distal *Id3* promoter to stimulate *Id3* expression. These observations were further extended to show that the mechanisms through which BMP2 regulates the *Id3* gene are likely conserved across cell types and in humans. Apart from their role in the gonadotropes, *Id*s are also expressed in multiple cell types. Id proteins are mainly involved in controlling the balance between

proliferation and differentiation, and thus identifying the transcriptional mechanism regulating the expression of Id proteins may aid in comprehending proliferative diseases such as pulmonary arterial hypertension (PAH) [481]. Understanding the mechanistic basis of diseases may aid in highlighting new drug targets.

5.1 Role of BMP2 receptors in regulating *Fshb* expression in LβT2

In Chapter 2, I demonstrated that BMP2 preferentially signals through BMPR1A and BMPR2 to stimulate *Fshb* transcription. Although BMP2 can also signal through BMPR1B, which is expressed in gonadotropes, the loss of BMPR1A cannot be compensated by BMPR1B. However, it is critical to establish roles for these receptors in gonadotrope function and FSH regulation *in vivo* (See Sections 5.5 and 5.6).

We considered the possibility that BMP2 may directly regulate *Fshb* gene expression through R-SMADs. However, BRE sequences are not present in the *Fshb* promoter, suggesting that BMP R-SMADs may not directly bind to the *Fshb* promoter to regulate transcription. Another possibility is that BMPs may act through SMAD-independent pathways to modulate *Fshb* expression [317, 419-421, 502-504]. Previous studies failed to detect an increase in p38 phosphorylation in response to BMP2 [282]; however, the involvement of other BMP-regulated MAP kinases has not yet been examined. To determine the involvement of SMAD-independent pathways, for example, the JNK pathway, we may first treat LβT2 cells with BMP2 and probe for the phosphorylation/activation of JNK. Provided that JNK is activated by BMP2, we may then treat LβT2 cells with SP600125, a small molecule inhibitor of the JNK pathway, and assess *Fshb* reporter activity in response to BMP2 alone or together with activin A.

Alternatively, *Fshb* transcriptional regulation may occur by an indirect mechanism. Previous studies in our lab suggest that BMP2 may indirectly affect *Fshb* transcription through the expression of other genes.

5.2 Ids as mediators of BMP2-stimulated *Fshb* expression

To identify BMP2 target genes in gonadotrope cells, I used cDNA microarray technology. Several *Id* genes, *Id1*, *Id2*, and *Id3*, appeared to be direct targets of BMP2. Although all three Ids when over-expressed can act with activin A or SMAD3 to regulate *Fshb* transcription (Chapter 3), only endogenous Id2 and Id3 are required to mediate the activin A and BMP2 synergism.

The *Id*s are known BMP responsive genes in a variety of cell types [223, 381-384, 387-388, 484-485] and have important roles in neurogenesis, angiogenesis, and bone formation [381-384, 387-388, 484-485]. The four Id sub-types (Id1-4) are located on different chromosomes, and exhibit similar, but not identical biological functions [381]; however, Id proteins display some functional redundancy *in vivo*. Although *Id1*-, *Id2*-, or *Id3*- knockout mice are viable, *Id1* and *Id3* double knockout mice die at day 13.5 of embryonic development [461]. These embryos exhibit small brain size, premature neuronal differentiation, and vascular abnormalities. This suggests that Id proteins are crucial for maintaining cell proliferation and differentiation, particularly in neuronal and vascular tissues. Ids are small proteins (13-20kDa) belonging to the helix-loop-helix (HLH) family of transcriptional regulators. bHLH transcription factors typically form homo- or hetero-dimers through their conserved HLH domains and regulate gene transcription by binding to their target promoter sequences through E-box *cis*-elements. Unlike other HLH proteins, Ids lack the basic amino acid domain necessary for DNA binding. Instead, Ids are conventionally observed to block transcriptional activity of bHLH proteins (such as Mash1, OLIG, NeuroD, and MyoD) by physically interacting with their HLH domains, forming DNA-binding-deficient hetero-dimers [384, 388, 484-485]. Many bHLH transcription factors are positive regulators of cell differentiation; therefore, in such cases, Id proteins serve as inhibitors of differentiation. For example, the E2A protein dimerizes with MyoD to activate genes promoting myoblast differentiation. BMPs stimulate Id1 protein expression, which competes with MyoD for heteodimerization with E2A. The lack of functional E2A-MyoD heterodimers favors an undifferentiated myoblast phenotype [505]. Similarly, in pancreatic AR42J cells, BMP4 stimulates Id2 expression, which binds to the bHLH transcription factor NeuroD. NeuroD is required for the differentiation of pancreatic islet cells; therefore, by blocking the differentiation pathway of these cells, Id2 promotes their expansion [485]. Conversely, BMP4-stimulated Id1 protein expression has been found to have antiproliferative effects in pulmonary arterial smooth muscle cells (PASMC) [481]. The exact mechanism by which Id1 inhibits cell proliferation has not yet been elucidated, but nonetheless emphasizes the cell-specific effects of Id proteins. Collectively, studies from different cell types show that Id proteins are important regulators of cell-specific biological functions by creating a balance between proliferation and differentiation [385, 387-388, 484-485, 506].

I demonstrated that SMAD3 and Id proteins can physically interact. Instead of blocking transcriptional activation, Ids seem to facilitate SMAD3's effect on *Fshb* transcription. The fact that Id proteins can bind to a non-bHLH transcription factor and promote its action rather than impair it, supports a mechanism that is divergent from Id's typical mode of action. The precise mechanism through which Ids produce their effects on *Fshb* transcription has not yet been elucidated; however,

several speculations can be made about their mechanisms of action. SMADs on their own bind weakly to DNA, but their affinity for DNA can be enhanced through interactions with specific co-factors [250, 443, 466-473]. Therefore, Ids might increase the affinity of SMAD3 for the *Fshb* promoter. Most SMAD-interacting co-factors have relatively high DNA binding affinities [250, 443, 466-473]. Although there is no evidence that Ids bind directly to DNA, they may be involved in the recruitment of additional transcription factors to the *Fshb* promoter, thus forming larger protein complexes to facilitate SMAD3-DNA binding without binding DNA directly. Another possibility is that a bHLH family member might interact with SMAD3, tonically inhibiting its activity at the *Fshb* promoter [474]. BMP2-mediated up-regulation of Id2 and Id3 might then compete for binding to SMAD3, relieving its repression. In future studies, we can use techniques such as yeast two-hybrid screening [507-508] or tandem affinity purification (TAP) [509-512] to identify bHLH proteins that may interact with SMAD3 in LβT2 cells.

5.3 Mechanisms mediating *Id1/2/3/4* expression

A mechanism for BMP-stimulated *Id1/ID1* expression has been described. Collectively, BMPs may act through BMPR1A, BMPR2 and SMAD1/5/4 to regulate *Id1* expression in murine endocardium and vascular smooth muscle cells [377, 475-482]. BMP responsive elements have also been described in the *Id1/ID1* promoter. Many BMP responsive genes are regulated through bipartite elements, consisting of a consensus BRE sequence, typically (T)GGCGGC, closely associated with one or more CAGA(C) boxes [378]. BMP-regulated R-SMADs can bind the BRE site, whereas the CAGA(C) box can bind SMAD4. These bipartite elements are conserved between all four of the *Id* genes. Two bipartite elements exist in the *Id1/ID1* gene and promoter [378]. One at ~-1kb which was previously described by Lopez-Rovira *et al.* and Korchynskyi *et al.* as a necessary response element for BMP2 to induce murine and human *Id1*/*ID1* transcription [377, 477, 483]. The second one, however, found at +3.3kb has not yet been examined.

Less is known about BMP-mediated *Id3* transcriptional regulation. We and others have shown that BMPs preferentially acts through BMPR1A, BMPR2, and the intracellular signaling proteins SMAD1, 5, and 4 to stimulate *Id3* expression ([476, 497] and Chapter 4). Two bipartite elements exist in the *Id3/ID3* gene body and promoter [378]. One at ~-3.0kb was shown to be essential for BMP4-stimulated human *Id3* transcription in ovarian cancer cells [379]. The other bipartite element located at ~+1kb in the *Id3* gene also contributes to *Id3* transcriptional regulation by acting synergistically with the distal bipartite element [379]. The region between -184 and -34 of the murine *Id3* promoter is essential in maintaining basal *Id3* promoter-reporter activity in C2C12 cells [487].

Results from my work in gonadotrope cells complement this study by showing that the region between -568 and -528 of the murine *Id3* promoter is required to confer minimal BMP2 responsiveness. In addition, I identified a novel six base-pair (bp) BRE, AAGATA, in the proximal murine *Id3* promoter that is essential for BMP2 to elicit *Id3* transcription in LβT2 cells. Although SMAD1 and SMAD5 do not seem to bind directly to this *cis*-element, SMAD5/4 induction of *Id3* transcription is dependent upon it. This six bp BRE is followed closely by a CAGA box, qualifying it as a potential bipartite element. However, I did not examine a role for this CAGA box. Finally, I demonstrated that this proximal bipartite element in the *Id3* promoter can regulate *Id3* transcription synergistically with the distal bipartite element at ~-3.0kb (see Chapter 4). The involvement of several BREs or bipartite elements may serve to fine-tune *Id3* transcriptional expression.

An unknown protein complex can bind to the proximal six bp BRE of the murine *Id3* promoter *in vitro* and mutations made to this site impair BMP2-responsivness in the *Id3* promoter-reporter. Though I did not identify the component proteins in my analysis, future experiments can be conducted to this end using a variety of methods. For example, one could employ an affinity chromatography approach [513], coupled with EMSAs and DNA precipitation (DNAP) assays to isolate this unknown protein complex from LβT2 nuclear extracts. The proteins within this complex can then be identified by mass spectrometry [514]. Alternatively, because the protein complex of interest was identified in EMSAs as a distinct band, we can directly excise this band from the gel and identify the proteins by mass spectrometry [515]. Finally, we may also use a yeast one-hybrid screen to identify proteins that may interact with our sequence of interest [507, 516]. Although we can identify the binding of this protein complex to the *Id3* promoter in EMSAs, it may not necessarily bind in LβT2 cells. Further, it is unknown whether the binding of this complex is required for the BMP response to occur. Therefore, upon identification of possible protein candidates, we must confirm their association with the predicted region of the *Id3* promoter in LβT2 cells by ChIP analyses and validate their importance in BMP2-stimulated *Id3* transcription in LβT2 cells by siRNA-mediated depletion of the endogenous protein(s).

Considerably less is known about *Id2* regulation than *Id3* regulation. A bipartite element was identified at ~-3.0kb of the *Id2/ID2* promoter a few years ago [378]. Recent studies in C2C12 cells showed that a -3.0kb *Id2* promoter-reporter is responsive to BMP6 [501], indicating that the *Id2* gene is transcriptionally regulated by BMPs. Through mutational analysis, Nakahiro *et al.* demonstrated that this bipartite element is required for BMP6 to induce *Id2* transcription. In addition, they showed direct binding of SMADs to this site [501]. These new data may help explain my experience with the *Id2* promoter. Because our *Id2* promoter-reporter construct only contained -1.5kb of the promoter, it

lacked the essential bipartite element, and thus it was inadequate to confer BMP2 responsiveness. In future experiments, we may obtain a longer *Id2* promoter-reporter and test if the mechanisms identified in the C2C12 cells are conserved in LβT2 cells. Early analysis of the *Id2* promoter identified several putative transcription factor binding sites within -1.0kb of the AUG initiation codon, including E-boxes and Sp1 consensus sequences [517]. My work with the proximal *Id2* promoter suggests that these sites are not sufficient for the induction of *Id2* transcription by BMP2. However, these sites may be necessary for maintaining basal *Id2* expression. The receptors through which BMPs induce *Id2* transcription have not yet been described; however, unlike *Id1* and *Id3*, *Id2* expression does not seem to be dependent on BMPR1A [476].

Id4 is the least studied of the four *Id* genes. *Id4* expression, together with *Id1*, *Id2*, and *Id3*, is up-regulated by BMP2 and BMP6 in several cell types [518-520]. However, in gonadotrope cells *Id4* was the only *Id* transcript not affected by BMP2 treatment (Chapter 3) suggesting that its expression and/or regulation in gonadotropes is distinct from the other *Id* genes. Indeed, bipartite elements are not present in the *Id4* promoter, although one exists within the *Id4* gene body itself [378]. Similar to *Id2*, initial analysis of the *Id4* promoter identified several putative transcription factor binding sites within -2.0kb upstream of the AUG initiation codon. These sites include E-boxes, a CREB binding site, an E2F site, and Sp1 sites [517]. The -48 to +32 region of the *Id4* promoter is the minimum sequence required to maintain basal *Id4* expression in HeLa cells [521]. Although BMPs can upregulate *Id4* expression in several cell types [518-520, 522], it has not yet been examined whether the *Id4* promoter is responsive to BMPs.

5.4 BMP2 and activin A synergism in primary pituitary and purified gonadotrope cultures

BMP2, 4, 6, and 7 stimulate *Fshb* transcription either alone or in synergy with activins [282, 401, 409]. Whereas *Bmp6* and *Bmp7* are readily detectable both in murine pituitaries and LβT2 cells [282, 401, 405], *Bmp2* and *Bmp4* are only detected in murine pituitaries [282]. This suggests that BMP2 and 4 are expressed in gonadotropes at very low levels, or are not expressed at all. Nonetheless, BMP2 and 4 stimulate *Fshb* transcription more potently than BMP6 and 7 in LβT2 cells. Furthermore, BMP2 and 4 can synergize with activins to regulate *Fshb* expression [282]. This suggests that BMP2 and 4 may act in a paracrine fashion to regulate *Fshb* expression. There are studies suggesting that BMP2 and BMP4 are produced endogenously in corticotropes [523] and somatotropes [524], which may then be secreted and thereby stimulate *Fshb* transcription in neighbouring gonadotropes. In future studies, this hypothesis can be tested by co-culturing LβT2 cells

together with AtT20 cells (a corticotrope cell line) or GH3 cells (a somatotrope cell line), and examining the *Fshb* reporter activity in the presence or absence of activin A treatment. To confirm the expression of BMP2 and 4 in corticotropes and somatotropes, and to examine their expression in other anterior pituitary cell types, such as thyrotropes and lactotropes, immunofluorescence can be performed on the anterior pituitary to co-localize the expression of BMP2 or 4 with cells expressing markers specific for the different anterior pituitary cell lineages. Interestingly, it was observed that BMP2/7 and BMP4/7 heterodimers can act more potently and effectively than their respective homodimers in certain contexts [354-355]. Because *Bmp2*, *Bmp4*, and *Bmp7* mRNA expression was detected in murine pituitaries, it is possible that such heterodimers of BMP may be an endogenous and more potent source of FSH stimulus [354-355, 401].

Based on the observation that BMP2 and activin A synergistically stimulate *Fshb* reporter activity in LβT2 cells, my thesis was designed to dissect BMP2's signaling pathway in these cells. As most, if not all, of my experiments were conducted in the immortalized gonadotrope cell line, I wished to confirm that the same phenomena exist in primary gonadotrope cells. Although the effects of BMP6 and BMP7 on *Fshb* mRNA expression were first reported in murine primary pituitary cultures, the corresponding effects of BMP2 and BMP4 have not been determined. I therefore treated primary murine pituitary cultures with activin A, BMP2, or both and measured *Fshb* mRNA expression by quantitative real-time PCR (qPCR) (Figure 5.2). As expected, activin A stimulated *Fshb* expression (~3-fold). BMP2 alone had no effect; but this may be due to the ligand concentrations used in my assays. Huang *et al*. detected an increase in *Fshb* mRNA transcripts in response to 1 µg/ml of BMP6 or BMP7 [401], whereas I only used 25 ng/ml of BMP2. Nonetheless, BMP2 and activin A together synergistically stimulated *Fshb* transcription (6-fold). Unfortunately, results from these experiments were generally inconsistent. That is, BMP2 enhanced the activin A response 43% of the time (3/7 individual experiments), inhibited the activin A response 14% of the time (1/7), or had no effect in 43% (3/7) of cases. The cause of these inconsistencies is not yet clear. The activin A and BMP2 synergism is evident in LβT2 cells; however, the appearance of this synergism in primary cultures is inconsistent. Recall that LβT2 cells represent a homogeneous population of immortalized gonadotropes, whereas primary pituitary cultures represent a mixture of various cell types. Thus, in addition to BMPs, *Fshb* transcription can be regulated by numerous paracrine regulators secreted from these cells. BMP antagonists, such as noggin, may be up-regulated in pituitary cultures to neutralize the effects of BMP2. BMPs can also act on other anterior pituitary cell types, for example, corticotropes and somatotropes, which also express BMP receptors [523-524]. It is not clear how gonadotropes respond to BMP2 stimulation in the presence of different neighbouring pituitary cells.

Exogenous BMP2 may act on any BMP-receptor-expressing pituitary cell, and change their secretion profile. This in turn may affect the balance of hormones and paracrine acting factors available in the extracellular space, thus affecting gonadotrope signaling and *Fshb* transcriptional regulation. Another area of future investigation could include determining which other cell types BMPs can act on in the anterior pituitary, how BMPs affect the secretion of different signaling factors from these cells, and whether these signaling factors have an effect on gonadotropes and *Fshb* expression. Currently however, inconsistent results prevent me from drawing any conclusions from primary pituitary culture experiments.

To determine the effect of BMP2 on primary gonadotropes in isolation, I performed several experiments in purified H2Kk gonadotropes (see Chapter 1, Section 3.1.5) (Figure 5.3). The stimulatory effect of exogenous ligands was greatly amplified in the purified gonadotropes. Activin A stimulated *Fshb* mRNA expression by 40-fold, BMP2 alone had no effect, but BMP2 together with activin A stimulated *Fshb* mRNA expression by 60-fold. Nonetheless, the synergism between BMP2 and activin A was similarly inconsistent in these cells. BMP2 enhanced the activin A response 40% (2/5) of the time, inhibited it 20% (1/5) of the time, and had no effect the remaining 40% (2/5) of the time. This variability in the BMP2 and activin A synergism is difficult to explain. Furthermore, the synergistic effects of BMP2 and activin A in primary cultures are not as potent as those seen in LβT2 cells. Overall, this suggests that additional studies are required to determine whether BMP2 is an important regulator of *Fshb* expression in gonadotrope cells.

5.5 The importance of endogenous activins and BMPs in *Fshb* expression

To test the importance of endogenous BMPs and activins in *Fshb* transcription, I cultured mixed (Figure 5.4) and purified gonadotrope primary cultures (Figure 5.5) with several physiological and pharmacological inhibitors. Because activins are known regulators of *Fshb* expression and FSH secretion [12-13], it was anticipated that SB431542, a small molecule inhibitor of ACVR1B, TGFBR1, and ACVR1C, would be a very potent inhibitor of basal *Fshb* expression. At 1 μM, *Fshb* expression was reduced to 10% of control (untreated cells), and with 10 μM of SB431542, *Fshb* expression was no longer detectable in mixed pituitary cultures. The inhibitory effect of SB431542 was also observed in purified gonadotropes. Follistatin, a physiological antagonist of activin, similarly reduced *Fshb* expression in mixed pituitary cultures. At 200 ng/ml follistatin, *Fshb* expression was reduced to 15% of control. Compound C, a small molecule inhibitor of ACVR1, BMPR1A, and BMPR1B (i.e., the BMP type I receptors), also inhibited basal *Fshb* expression. With 2 μM of Compound C, *Fshb* expression was reduced to 20% of control in mixed cultures. The

inhibitory effect of Compound C was also observed in purified gonadotropes. Finally noggin, a physiological antagonist of BMPs, was also found to inhibit *Fshb* expression in mixed pituitary cultures. The effects of exogenous inhibitors were consistent, and suggested that *Fshb* expression is tonically regulated by endogenous BMPs and activins. Although the synergism between exogenous BMP2 and activin A was neither confirmed nor refuted in primary cultures, I demonstrated a role for endogenous BMPs in the regulation of basal *Fshb* expression in both whole pituitary and purified gonadotrope cultures. It is possible that *Fshb* expression may be more effectively stimulated by BMP subtypes other than BMP2, perhaps BMPs that are endogenously expressed in gonadotropes. Future studies should include testing the effect of activin A with BMP6 or BMP7 on *Fshb* expression in whole pituitary or purified gonadotrope primary cultures.

5.6 Knocking out BMPR1A in primary pituitary cultures

In Chapter 2, I demonstrated that BMP2 acts through BMPR1A to regulate *Fshb* transcription, and in Chapter 4, I showed that BMP2 acts through the same receptor to induce *Id3* expression. To determine the importance of *Bmpr1a* in murine gonadotropes, I examined the effects of *Bmpr1a* ablation using the Cre/*loxP* system. The Cre/*loxP* system allows one to study the effect of gene deletion in a specific tissue or cell type. The introduction of two consensus *loxP* sequences in a target gene should not affect its function. However, when Cre recombinase is expressed, it recognizes the two *loxP* sites in the same orientation and excises the sequences in between them, thus inactivating the target gene [525-526]. *Bmpr1a*$^{flox/flox}$ mice, provided by Dr. Yuji Mishina (University of Michigan, Michigan, USA) [488], were generated such that exon 2 of their *Bmpr1a* gene is flanked with *loxP* sites. Whole pituitary primary cultures from these mice were infected with adenovirus expressing GFP or Cre-IRES-GFP. After 24 hrs of infection, cells were treated with activin A, BMP2, or both. Cells were then harvested after 24 hrs and *Fshb* mRNA levels measured by qPCR. As shown in Chapter 4, analysis of genomic DNA confirmed successful recombination of the *Bmpr1a* gene, with an associated decrease of *Bmpr1a* mRNA levels in primary pituitary cultures (Figure S4.3). The knockdown of *Bmpr1a* abrogated BMP2-induced *Id3* expression (Chapter 4), suggesting a crucial role for this receptor in BMP2-mediated *Id3* induction. However, the knockdown of *Bmpr1a* had no effect on *Fshb* expression in response to activin A, BMP2, or activin A plus BMP2 (data not shown). As in primary cultures from wild-type mice, BMP2 had no effect on *Fshb* mRNA expression in primary cultures from *Bmpr1a* $^{flox/flox}$ mice. Importantly, the BMP2 and activin A synergism could not be detected in these cultures. This suggests that BMP2 may not stimulate *Fshb* expression in pituitary cultures from *Bmpr1a* $^{flox/flox}$ mice. It may therefore not be surprising that the knockout of *Bmpr1a* in these cultures had no effect on *Fshb* expression in response to BMP2. That is, there is no effect to

antagonize. Whether this lack in activin A and BMP2 synergism is specific to *Bmpr1a* $^{flox/flox}$ mice is still undetermined.

Discrepancies between cell lines and primary cultures may be due to differences in the intracellular milieu between immortalized cells grown over many passages and normal cells extracted from animals. The process of immortalization may also change the physiology of the cell. Furthermore, as mentioned previously, gonadotropes in primary cultures are grown together with other cell types that may secrete factors modulating the functions of gonadotropes which may change the way they respond to different stimuli and the way *Fshb* transcription is controlled.

5.7 Conditional ablation of BMPR1A in gonadotropes of mice

To definitively determine a role for BMPR1A in *Fshb* expression, we must knock out *Bmpr1a* in the gonadotropes of mice, and examine the effect on *Fshb* expression and fertility *in vivo*. We have already generated these gonadotrope-specific *Bmpr1a* knockout mice by crossing the *Bmpr1a* $^{flox/flox}$ mice [488] with the *Gnrhr*-Cre (GRIC) transgenic mice [527], and confirmed by PCR that recombination has occurred in the pituitaries. Next, *Bmpr1a* and *Fshb* expression in the pituitaries of these animals will be assessed by qPCR, and circulating levels of FSH will be measured by enzyme-linked immunosorbent assay (ELISA). The reproductive parameters of *Bmpr1a* ablation *in vivo* will also be assessed, including the ovary weight, the litter size, the frequency of pregnancy, and the number of maturing follicles in the ovaries of these female mice. If BMPs act specifically through BMPR1A to stimulate *Fshb* transcription one would expect the knockout of *Bmpr1a* to hinder BMP signaling in gonadotropes, resulting in decreased pituitary *Fshb* mRNA and circulating FSH protein levels. Depending on the extent of *Fshb* transcriptional inhibition, ovarian functions of these mice may be affected leading to a decrease in the numbers of mature follicles, which will ultimately decrease the litter size and frequency of litter production. Because knocking out the *Fshb* gene itself does not have a dramatic impact on spermatogenesis or fertility in male mice [114], one would not predict the *Bmpr1a* deletion, even if it does affect *Fshb* production, to have much impact on the male reproductive capacity. However, if the knockout of *Bmpr1a* significantly reduces *Fshb* mRNA/FSH protein expression in males, one may observe a decrease in testis weight, Sertoli cell number, and sperm count. Although these are important of experiments being conducted in the lab, the generation of these mice is beyond the scope of my thesis, and I will not be assessing the phenotypes of these mice.

In vivo knockout of *Bmpr1a* may determine the importance of *Bmpr1a* in the regulation of *Fshb* expression once and for all. However, if the knockout of *Bmpr1a* has no effect on *Fshb* expression we

cannot completely rule out the involvement of BMPs in *Fshb* transcriptional regulation. We must then examine whether other BMP receptors can compensate for the loss of BMPR1A. BMP2 has a high affinity for both BMPR1A and BMPR1B [363]. However, the expression of *Bmpr1b* is considerably lower than *Bmpr1a* in gonadotropes [282, 409], which may explain the lack of compensation by BMPR1B during the transient knockdown of *Bmpr1a* in LβT2 cells (Chapter 2). Long-term knockdown of *Bmpr1a in vivo* may allow enough time for gonadotropes to up-regulate *Bmpr1b* expression and compensate for the loss of *Bmpr1a* and thereby preserve *Fshb* expression. *In vitro* analysis suggests that *Bmpr1a* mediates BMP2-stimulated *Id3* expression in gonadotrope cells (Chapter 4). In addition to examining the effects of *Bmpr1a* ablation on *Fshb* expression, we may similarly determine the importance of this receptor on *Id3* transcriptional regulation by measuring *Id3* mRNA and Id3 protein expression in the pituitaries of these mice.

Conclusion

Unveiling of the mechanisms controlling *Fshb* transcription has been a slow and challenging process. For a long time, the lack of appropriate models and tools, including homologous cell lines, made it difficult to study the transcriptional regulation of the *Fshb* subunit. Although the recent development of LβT2 cells has greatly advanced our knowledge of *Fshb* transcriptional regulation, it remains the only homologous cell line available. The development of a human immortalized gonadotrope cell line would be ideal, particularly because *Fshb/FSHB* transcriptional regulation seems to vary greatly between species. Experiments using current protocols for purifying gonadotropes still face some difficulties; for example, the amount of extracted gonadotropes from these mice is still relatively small, making them difficult to work with. The development of transgenic mice which can further improve the yield of pure gonadotropes may provide future possibilities in advancing our understanding of *Fshb* regulation. Nevertheless, animal models will be required to validate the models generated from *in vitro* data. In comparison to a few decades ago, the understanding of *Fshb* transcriptional regulation has advanced considerably; however, the field is still young and many questions still remain unanswered. Continuing development of tools and cell lines will further help us understand how *Fshb* is regulated.

The results I present here contribute to knowledge in the field of *Fshb* transcriptional regulation. My thesis describes, in part, the mechanisms by which BMP2 regulates *Fshb* expression in synergy with activin A. I have demonstrated that BMPR1A is the required BMP-type I receptor for BMP2 to stimulate *Fshb* expression synergistically with activin A in LβT2 cells. However, ablation of *Bmpr1a* in gonadotropes of primary pituitary cultures was unable to confirm this model because the BMP2

and activin A synergism was not observed in primary cultures from these animals. In fact, the BMP2 and activin A synergism was inconsistent even in primary cultures from wild-type animals, and thus it is possible that the requisite conditions for culturing primary cells still require optimization. Nonetheless, primary pituitary cultures from wild-type mice and gonadotrope cultures from H2Kk mice suggest that endogenous BMPs may tonically regulate *Fshb* expression, similar to endogenous activin B. The involvement of BMPR1A and BMPs will become clearer with the analysis of gonadotrope-specific *Bmpr1a* knockout mice.

I identified the Id proteins as BMP2 targets in gonadotropes and found that Id2 and Id3 have a role in stimulating *Fshb* expression cooperatively with activin A. BMP2 and activin A synergism may integrate at the level of Id2/3 and SMAD3, as a physical interaction was observed between them. However, the exact mechanism by which Id2/3 regulates *Fshb* transcription has yet to be determined. The increase in *Id3* transcription in response to BMP2 is dependent on the BMP receptors BMPR1A and BMPR2. BMP2-mediated *Id3* expression is also dependent on a proximal BRE and the intracellular signaling proteins SMAD1/5/4, though SMAD1/5/4 does not appear to bind this BRE. A protein complex was observed to bind the proximal BRE; however, its composition has not been determined and a necessary role for it in BMP2-mediated transcriptional activation has not been confirmed. Nonetheless, maximal *Id3* promoter activation is dependent on both the proximal BRE and a previously identified distal BRE; the findings of which were consistently observed between species and cell types.

In summary, FSH is crucial for controlling gametogenesis and steroidogenesis, and its expression is tightly regulated by a variety of endocrine, paracrine, and autocrine factors. The novel observation that BMPs may be involved in *Fshb* transcriptional regulation sparked much attention among those studying the TGFβ superfamily ligand signaling in the pituitary. The addition of yet another level of *Fshb* transcriptional regulation may allow more precise fine-tuning of *Fshb* expression. Furthermore, BMPs specifically regulate *Fshb* transcription, while not affecting *Lhb* expression, suggesting that in addition to activins, BMPs may be additional factors contributing to differential regulation of FSH and LH expression. The understanding of the mechanisms mediating BMP-induced *Fshb* transcription is important. As such, further *in vivo* investigation into the roles and mechanisms of BMPs in regulating *Fshb* transcription is required.

Figure legends

Figure 5.1: Summary of findings. Partial mechanism by which BMP2 regulates *Fshb* transcription in conjunction with activin A.

Figure 5.2: Pituitary cultures from wild-type mice were pre-treated with 10 µM SB431542 and then treated with 25 ng/ml activin A, 50 ng/ml BMP2, or both ligands. Cells were harvested after 24 h and changes in *Fshb* mRNA expression were measured by qRT-PCR. Data are presented relative to untreated cultures. Treatments were performed in triplicate but cells were pooled for analysis, n=1. Figure shows one experiment.

Figure 5.3: Gonadotropes purified from H2Kk transgenic mice were pre-treated with 10µM SB431542 and then treated with 25 ng/ml activin A, 50ng/ml BMP2, or both ligands. Cells were harvested after 24 h and changes in *Fshb* mRNA expression were measured by qRT-PCR. Data are presented relative to untreated cultures. Treatments were performed in triplicate but cells were pooled for analysis, n=1. Figure shows one experiment.

Figure 5.4: A) Pituitary cultures from wild-type mice were treated with increasing concentrations of SB431542 or follistatin. B) Pituitary cultures from wild-type mice were treated with increasing concentrations of Compound C or noggin. In both panels, cells were harvested after 24 h and changes in *Fshb* mRNA expression were measured by qRT-PCR. Data are presented relative to untreated cultures. Each treatment was performed in 9 wells, but cells from 3 wells were pooled for analysis, n=3. Figure shows one experiment.

Figure 5.5: Gonadotropes purified from H2Kk transgenic mice were treated with 10 µM SB431542, 10µM Compound C, or 25 ng/ml activin A. Cells were harvested after 24 h and changes in *Fshb* mRNA expression were measured by qRT-PCR. Data are presented relative to untreated cultures. Treatments were performed in triplicate but cells were pooled for analysis, n=1. Figure shows one experiment.

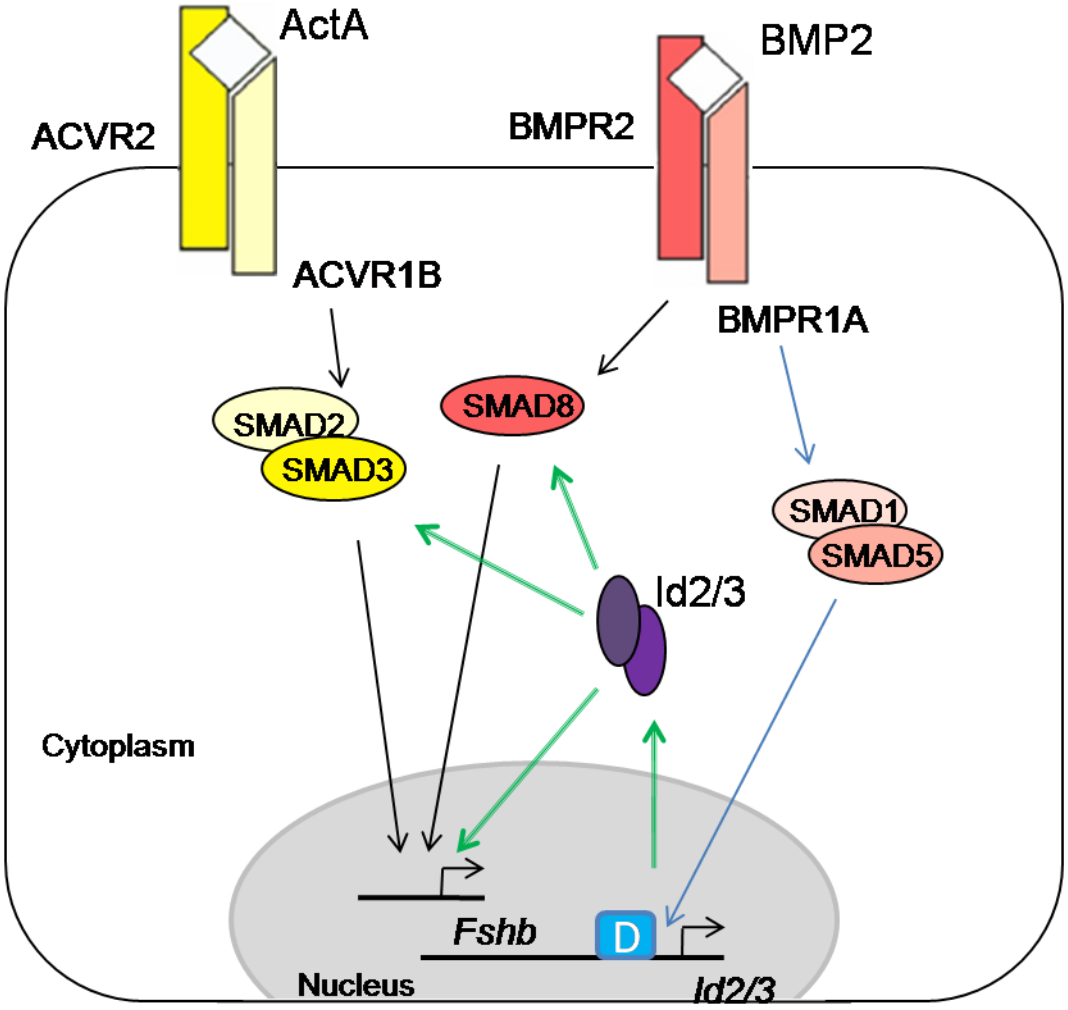

ActA
ACVR2
BMP2
BMPR2
ACVR1B
BMPR1A
SMAD2
SMAD3
SMAD8
SMAD1
SMAD5
Id2/3
Cytoplasm
Fshb
D
Nucleus
Id2/3

Figure 5.1

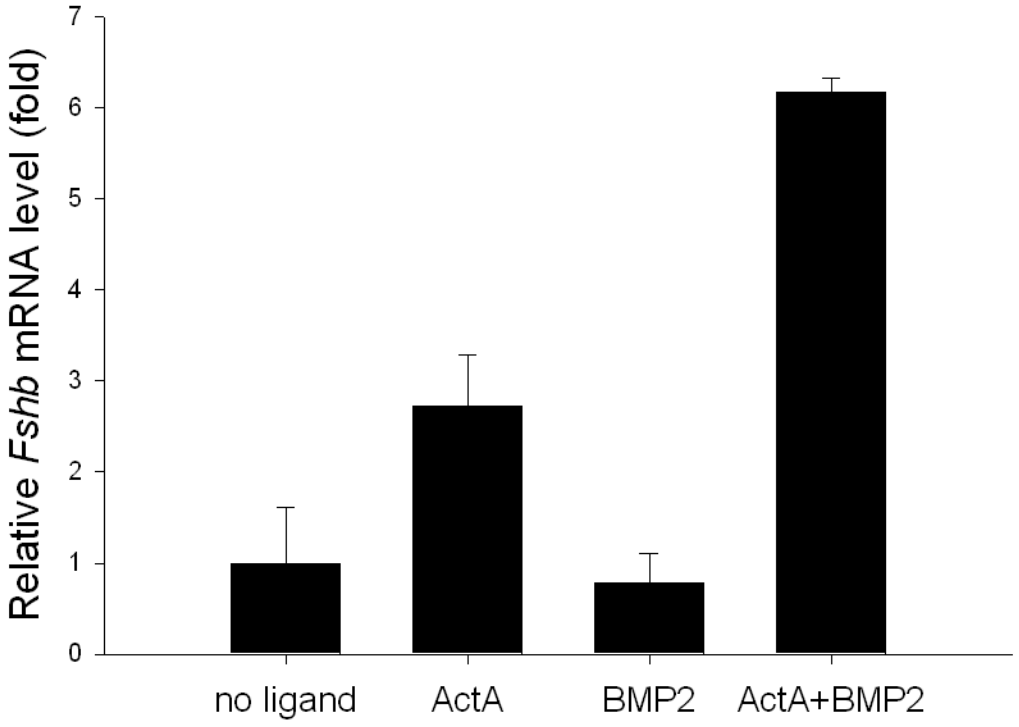

Relative Fshb mRNA level (fold)
7
6
5
4
3
2
1
0
no ligand
ActA
BMP2
ActA+BMP2

Figure 5.2

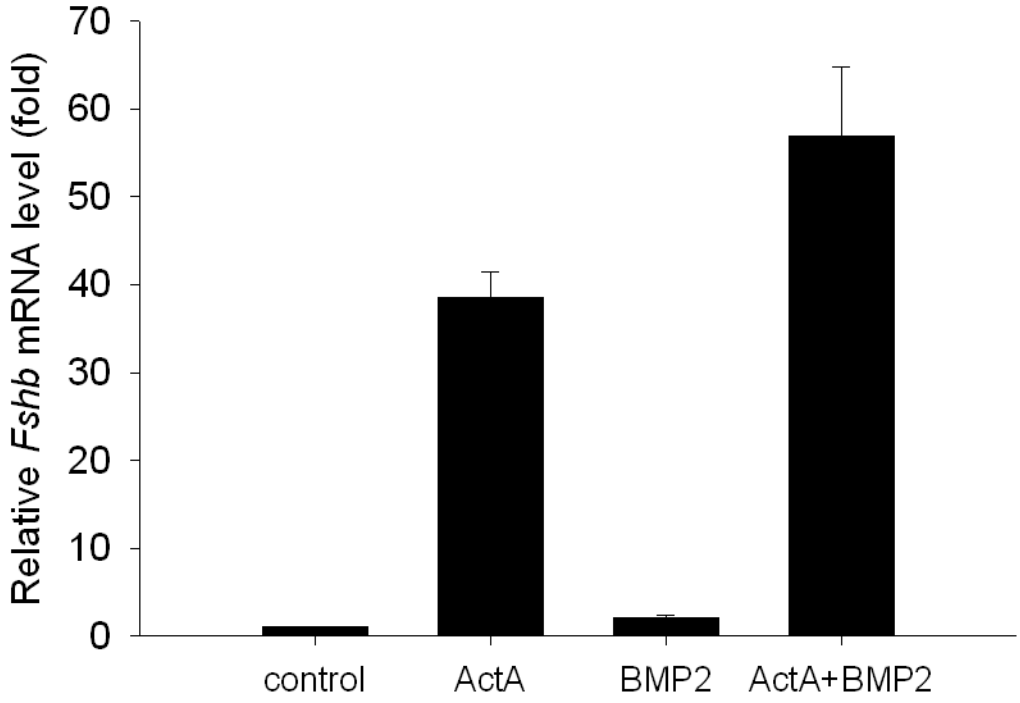

Relative Fshb mRNA level (fold)
70
60
50
40
30
20
10
0
control
ActA
BMP2
ActA+BMP2

Figure 5.3

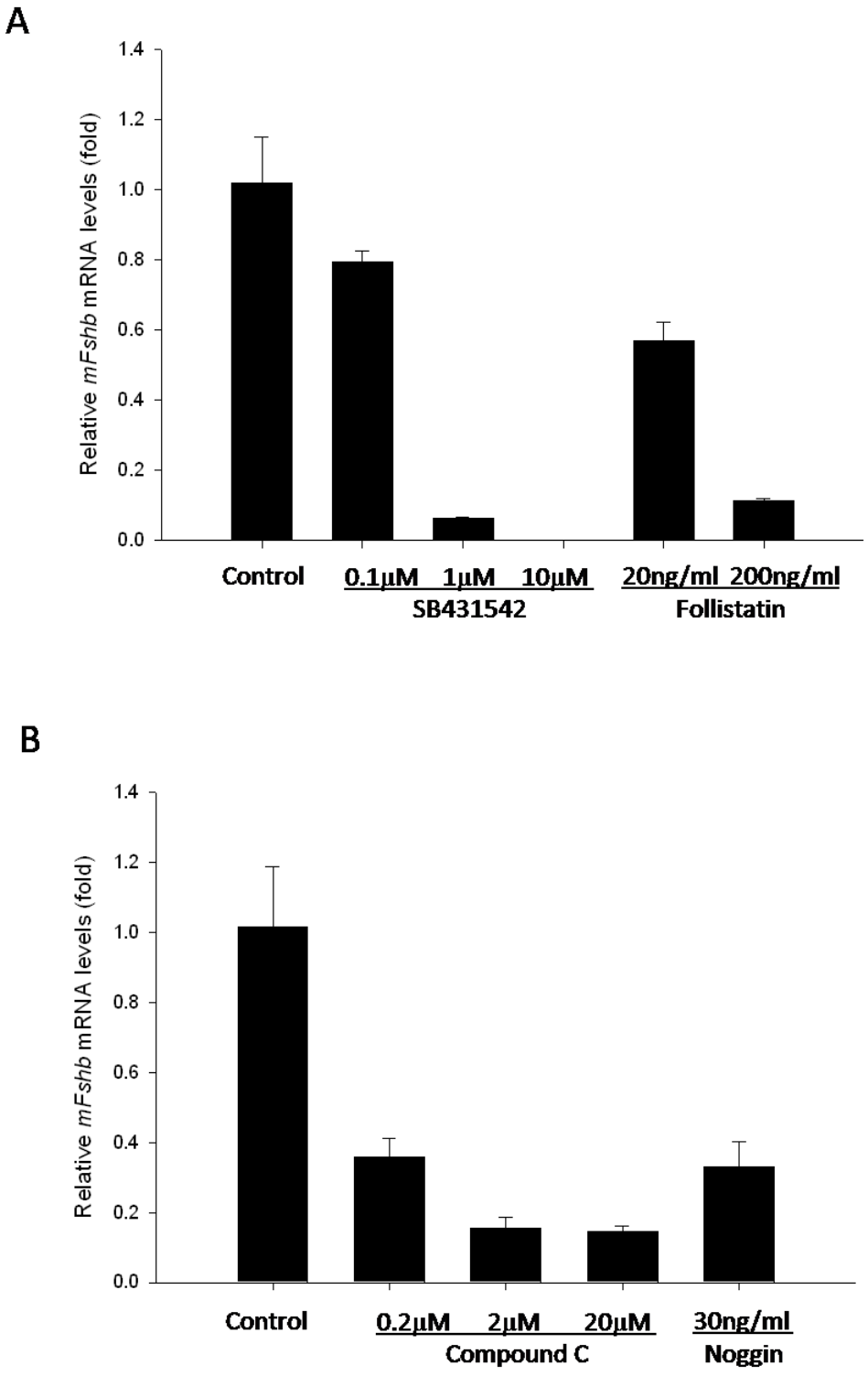

A
Relative mFshb mRNA levels (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.1μM
1μM
10μM
SB431542
20ng/ml
200ng/ml
Follistatin
B
Relative mFshb mRNA levels (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.2μM
2μM
20μM
Compound C
30ng/ml
Noggin

Figure 5.4

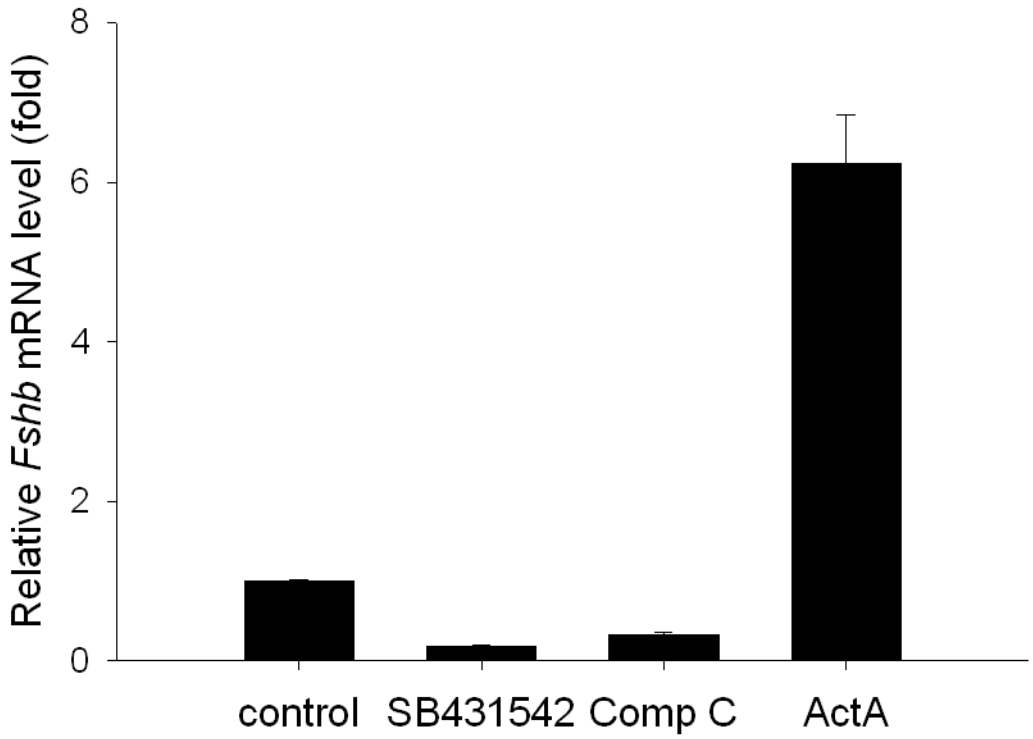

Relative Fshb mRNA level (fold)
8
6
4
2
0
control
SB431542
Comp C
ActA

Figure 5.5

References

1. Marshall, P.E. and P.C. Goldsmith, *Neuroregulatory and neuroendocrine GnRH pathways in the hypothalamus and forebrain of the baboon.* Brain Res, 1980. **193**(2): p. 353-72.
2. Jansen, H.T., et al., *Identification and distribution of neuroendocrine gonadotropin-releasing hormone neurons in the ewe.* Biol Reprod, 1997. **56**(3): p. 655-62.
3. Kaiser, U.B., et al., *Differential effects of gonadotropin-releasing hormone (GnRH) pulse frequency on gonadotropin subunit and GnRH receptor messenger ribonucleic acid levels in vitro.* Endocrinology, 1997. **138**(3): p. 1224-31.
4. Weiss, J., et al., *Divergent responses of gonadotropin subunit messenger RNAs to continuous versus pulsatile gonadotropin-releasing hormone in vitro.* Mol Endocrinol, 1990. **4**(4): p. 557-64.
5. Belchetz, P.E., et al., *Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone.* Science, 1978. **202**(4368): p. 631-3.
6. Hamernik, D.L. and T.M. Nett, *Gonadotropin-releasing hormone increases the amount of messenger ribonucleic acid for gonadotropins in ovariectomized ewes after hypothalamic-pituitary disconnection.* Endocrinology, 1988. **122**(3): p. 959-66.
7. Ferris, H.A. and M.A. Shupnik, *Mechanisms for pulsatile regulation of the gonadotropin subunit genes by GNRH1.* Biol Reprod, 2006. **74**(6): p. 993-8.
8. Marshall, J.C., et al., *GnRH pulses--the regulators of human reproduction.* Trans Am Clin Climatol Assoc, 1993. **104**: p. 31-46.
9. Bilezikjian, L.M., et al., *Pituitary actions of ligands of the TGF-beta family: activins and inhibins.* Reproduction, 2006. **132**(2): p. 207-15.
10. Schwall, R., et al., *Multiple actions of recombinant activin-A in vivo.* Endocrinology, 1989. **125**(3): p. 1420-3.
11. Rivier, C. and W. Vale, *Effect of recombinant activin-A on gonadotropin secretion in the female rat.* Endocrinology, 1991. **129**(5): p. 2463-5.
12. Bilezikjian, L.M., J.M. Vaughan, and W.W. Vale, *Characterization and the regulation of inhibin/activin subunit proteins of cultured rat anterior pituitary cells.* Endocrinology, 1993. **133**(6): p. 2545-53.
13. Muttukrishna, S. and P.G. Knight, *Inverse effects of activin and inhibin on the synthesis and secretion of FSH and LH by ovine pituitary cells in vitro.* J Mol Endocrinol, 1991. **6**(2): p. 171-8.
14. Sjogren, A., T. Hillensjo, and L. Hamberger, *The regulatory role of FSH in steroid formation by luteinized human granulosa cells in culture.* Hum Reprod, 1987. **2**(6): p. 469-73.
15. Bergh, C., et al., *Steroid production in cultured thecal cells obtained from human ovarian follicles.* Hum Reprod, 1993. **8**(4): p. 519-24.
16. Shaw, N.D., et al., *Estrogen negative feedback on gonadotropin secretion: evidence for a direct pituitary effect in women.* J Clin Endocrinol Metab, 2010. **95**(4): p. 1955-61.
17. Gill, S., et al., *Evidence that GnRH decreases with gonadal steroid feedback but increases with age in postmenopausal women.* J Clin Endocrinol Metab, 2002. **87**(5): p. 2290-6.
18. Welt, C.K., et al., *Control of follicle-stimulating hormone by estradiol and the inhibins: critical role of estradiol at the hypothalamus during the luteal-follicular transition.* J Clin Endocrinol Metab, 2003. **88**(4): p. 1766-71.
19. Gregory, S.J. and U.B. Kaiser, *Regulation of gonadotropins by inhibin and activin.* Semin Reprod Med, 2004. **22**(3): p. 253-67.
20. Welt, C.K., *Regulation and function of inhibins in the normal menstrual cycle.* Semin Reprod Med, 2004. **22**(3): p. 187-93.

21. Depaolo, L.V., M. Shimonaka, and N. Ling, *Regulation of pulsatile gonadotropin secretion by estrogen, inhibin, and follistatin (activin-binding protein) in ovariectomized rats.* Biol Reprod, 1992. **46**(5): p. 898-904.
22. Rivier, C., J. Rivier, and W. Vale, *Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat.* Science, 1986. **234**(4773): p. 205-8.
23. Rivier, C., et al., *Effect of recombinant inhibin on gonadotropin secretion during proestrus and estrus in the rat.* Endocrinology, 1991. **128**(5): p. 2223-8.
24. Rivier, C., et al., *Effect of recombinant inhibin on luteinizing hormone and follicle-stimulating hormone secretion in the rat.* Endocrinology, 1991. **128**(3): p. 1548-54.
25. Rivier, C. and W. Vale, *Effect of recombinant inhibin on follicle-stimulating hormone secretion by the female rat: interaction with a gonadotropin-releasing hormone antagonist and estrogen.* Endocrinology, 1991. **129**(4): p. 2160-5.
26. Carroll, R.S., et al., *In vivo regulation of FSH synthesis by inhibin and activin.* Endocrinology, 1991. **129**(6): p. 3299-304.
27. Burger, H.G., *Evidence for a negative feedback role of inhibin in follicle stimulating hormone regulation in women.* Hum Reprod, 1993. **8 Suppl 2**: p. 129-32.
28. Widmaier, E.P., H. Raff, and K.T. Strang, *Reproduction*, in *Vander, Sherman, & Luciano's Human Physiology: The Mechanisms of Body Functions*. 2004, The McGraw-Hill Companies: New York, NY, USA. p. 643-694.
29. Porterfield, S.P., *Male/Female Reproduction*, in *Endocrine Physiology*. 2001, Mosby, Inc: St. Louis, Missouri, USA. p. 153-200.
30. Bendsen, E., et al., *Number of germ cells and somatic cells in human fetal ovaries during the first weeks after sex differentiation.* Hum Reprod, 2006. **21**(1): p. 30-5.
31. Baker, T.G. and L.L. Franchi, *The fine structure of oogonia and oocytes in human ovaries.* J Cell Sci, 1967. **2**(2): p. 213-24.
32. Fortune, J.E., et al., *The primordial to primary follicle transition.* Mol Cell Endocrinol, 2000. **163**(1-2): p. 53-60.
33. Craig, J., et al., *Gonadotropin and intra-ovarian signals regulating follicle development and atresia: the delicate balance between life and death.* Front Biosci, 2007. **12**: p. 3628-39.
34. Fortune, J.E., *The early stages of follicular development: activation of primordial follicles and growth of preantral follicles.* Anim Reprod Sci, 2003. **78**(3-4): p. 135-63.
35. McNatty, K.P., et al., *Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective.* Soc Reprod Fertil Suppl, 2007. **64**: p. 55-68.
36. Richards, J.S., *Perspective: the ovarian follicle--a perspective in 2001.* Endocrinology, 2001. **142**(6): p. 2184-93.
37. McGee, E.A. and A.J. Hsueh, *Initial and cyclic recruitment of ovarian follicles.* Endocr Rev, 2000. **21**(2): p. 200-14.
38. Garzo, V.G. and J.H. Dorrington, *Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin.* Am J Obstet Gynecol, 1984. **148**(5): p. 657-62.
39. Seifer, D.B., et al., *Differential secretion of dimeric inhibin in cultured luteinized granulosa cells as a function of ovarian reserve.* J Clin Endocrinol Metab, 1996. **81**(2): p. 736-9.
40. McLachlan, R.I., et al., *Circulating immunoreactive inhibin levels during the normal human menstrual cycle.* J Clin Endocrinol Metab, 1987. **65**(5): p. 954-61.
41. Ross, G.T., et al., *Pituitary and gonadal hormones in women during spontaneous and induced ovulatory cycles.* Recent Prog Horm Res, 1970. **26**: p. 1-62.
42. Filicori, M., et al., *Stimulation and growth of antral ovarian follicles by selective LH activity administration in women.* J Clin Endocrinol Metab, 2002. **87**(3): p. 1156-61.
43. Korenman, S.G. and B.M. Sherman, *Further studies of gonadotropin and estradiol secretion during the preovulatory phase of the human menstrual cycle.* J Clin Endocrinol Metab, 1973. **36**(6): p. 1205-9.

44. Howland, B.E., C. Faiman, and T.M. Butler, *Serum levels of FSH and LH during the menstrual cycle of the chimpanzee.* Biol Reprod, 1971. **4**(1): p. 101-5.
45. Xia, L., et al., *A surge of gonadotropin-releasing hormone accompanies the estradiol-induced gonadotropin surge in the rhesus monkey.* Endocrinology, 1992. **131**(6): p. 2812-20.
46. Caraty, A., et al., *Nature and bioactivity of gonadotropin-releasing hormone (GnRH) secreted during the GnRH surge.* Endocrinology, 1995. **136**(8): p. 3452-60.
47. Roseff, S.J., et al., *Dynamic changes in circulating inhibin levels during the luteal-follicular transition of the human menstrual cycle.* J Clin Endocrinol Metab, 1989. **69**(5): p. 1033-9.
48. Robertson, D.M., et al., *Comparison of inhibin immunological and in vitro biological activities in human serum.* J Clin Endocrinol Metab, 1988. **67**(3): p. 438-43.
49. Baird, D.T., et al., *Inhibin and oestradiol in the control of FSH secretion in the sheep.* J Reprod Fertil Suppl, 1991. **43**: p. 125-38.
50. le Nestour, E., et al., *Role of estradiol in the rise in follicle-stimulating hormone levels during the luteal-follicular transition.* J Clin Endocrinol Metab, 1993. **77**(2): p. 439-42.
51. Lenton, E.A., et al., *The human menstrual cycle: plasma concentrations of prolactin, LH, FSH, oestradiol and progesterone in conceiving and non-conceiving women.* J Reprod Fertil, 1982. **65**(1): p. 131-9.
52. Haisenleder, D.J., et al., *Inhibin secretion during the rat estrous cycle: relationships to FSH secretion and FSH beta subunit mRNA concentrations.* Life Sci, 1990. **47**(19): p. 1769-73.
53. Butcher, R.L., W.E. Collins, and N.W. Fugo, *Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat.* Endocrinology, 1974. **94**(6): p. 1704-8.
54. Levine, J.E., *New concepts of the neuroendocrine regulation of gonadotropin surges in rats.* Biol Reprod, 1997. **56**(2): p. 293-302.
55. Brown-Grant, K. and G. Raisman, *Abnormalities in reproductive function associated with the destruction of the suprachiasmatic nuclei in female rats.* Proc R Soc Lond B Biol Sci, 1977. **198**(1132): p. 279-96.
56. Coen, C.W. and P.C. MacKinnon, *Lesions of the suprachiasmatic nuclei and the serotonin-dependent phasic release of luteinizing hormone in the rat: effects on drinking rhythmicity and on the consequences of preoptic area stimulation.* J Endocrinol, 1980. **84**(2): p. 231-6.
57. Gray, G.D., et al., *Effects of lesions in various structures of the suprachiasmatic-preoptic region on LH regulation and sexual behavior in female rats.* Neuroendocrinology, 1978. **25**(3): p. 174-91.
58. Goodman, R.L. and E. Knobil, *The sites of action of ovarian steroids in the regulation of LH secretion.* Neuroendocrinology, 1981. **32**(1): p. 57-63.
59. Sarkar, D.K., et al., *Gonadotropin-releasing hormone surge in pro-oestrous rats.* Nature, 1976. **264**(5585): p. 461-3.
60. McClintock, J.A. and N.B. Schwartz, *Changes in pituitary and plasma follicle stimulating hormone concentrations during the rat estrous cycle.* Endocrinology, 1968. **83**(3): p. 433-41.
61. Hoak, D.C. and N.B. Schwartz, *Blockade of recruitment of ovarian follicles by suppression of the secondary surge of follicle-stimulating hormone with porcine follicular field.* Proc Natl Acad Sci U S A, 1980. **77**(8): p. 4953-6.
62. Uchida, K., M. Kadowaki, and T. Miyake, *Ovarian secretion of progesterone and 20alpha-hydroxypregn-4-en-3-one during rat estrous cycle in chronological relation to pituitary release of luteinizing hormone.* Endocrinol Jpn, 1969. **16**(2): p. 227-37.
63. Barraclough, C.A., et al., *Temporal interrelationships between plasma LH, ovarian secretion rates and peripheral plasma progestin concentrations in the rat: effects of Nembutal and exogenous gonadotropins.* Endocrinology, 1971. **88**(6): p. 1437-47.
64. Butcher, R.L., W.E. Collins, and N.W. Fugo, *Altered secretion of gonadotropins and steroids resulting from delayed ovulation in the rat.* Endocrinology, 1975. **96**(3): p. 576-86.

65. Rotello, R.J., et al., *Characterization of uterine epithelium apoptotic cell death kinetics and regulation by progesterone and RU 486.* Am J Pathol, 1992. **140**(2): p. 449-56.
66. Feng, Z., et al., *Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland.* J Cell Biol, 1995. **131**(4): p. 1095-103.
67. Juengel, J.L., et al., *Apoptosis during luteal regression in cattle.* Endocrinology, 1993. **132**(1): p. 249-54.
68. Knox, K.L. and N.B. Schwartz, *RU486 blocks the secondary surge of follicle-stimulating hormone in the rat without blocking the drop in serum inhibin.* Biol Reprod, 1992. **46**(2): p. 220-5.
69. Tebar, M., et al., *Effects of progesterone on the secondary surge of follicle-stimulating hormone in the rat.* Biol Reprod, 1997. **57**(1): p. 77-84.
70. Szabo, M., et al., *Mechanism of the inhibitory action of RU486 on the secondary follicle-stimulating hormone surge.* Endocrinology, 1996. **137**(1): p. 85-9.
71. Bowen, J.M., et al., *Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages.* Biol Reprod, 1996. **54**(5): p. 1120-7.
72. Gaytan, F., et al., *Role of prolactin in the regulation of macrophages and in the proliferative activity of vascular cells in newly formed and regressing rat corpora lutea.* Biol Reprod, 1997. **57**(2): p. 478-86.
73. Rothchild, I., *The corpus luteum revisited: are the paradoxical effects of RU486 a clue to how progesterone stimulates its own secretion?* Biol Reprod, 1996. **55**(1): p. 1-4.
74. Gaytan, F., et al., *Both prolactin and progesterone in proestrus are necessary for the induction of apoptosis in the regressing corpus luteum of the rat.* Biol Reprod, 1998. **59**(5): p. 1200-6.
75. Kuopio, T., L.J. Pelliniemi, and I. Huhtaniemi, *Rapid Leydig cell proliferation and luteinizing hormone receptor replenishment in the neonatal rat testis after a single injection of human chorionic gonadotropin.* Biol Reprod, 1989. **40**(1): p. 135-43.
76. Teerds, K.J., et al., *Proliferation and differentiation of possible Leydig cell precursors after destruction of the existing Leydig cells with ethane dimethyl sulphonate: the role of LH/human chorionic gonadotrophin.* J Endocrinol, 1989. **122**(3): p. 689-96.
77. Ramaswamy, S., T.M. Plant, and G.R. Marshall, *Pulsatile stimulation with recombinant single chain human luteinizing hormone elicits precocious sertoli cell proliferation in the juvenile male rhesus monkey (Macaca mulatta).* Biol Reprod, 2000. **63**(1): p. 82-8.
78. Buzzard, J.J., N.G. Wreford, and J.R. Morrison, *Marked extension of proliferation of rat Sertoli cells in culture using recombinant human FSH.* Reproduction, 2002. **124**(5): p. 633-41.
79. Simpson, B.J., F.C. Wu, and R.M. Sharpe, *Isolation of human Leydig cells which are highly responsive to human chorionic gonadotropin.* J Clin Endocrinol Metab, 1987. **65**(3): p. 415-22.
80. Tesarik, J., et al., *Human spermatogenesis in vitro: respective effects of follicle-stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis.* J Clin Endocrinol Metab, 1998. **83**(12): p. 4467-73.
81. Hayes, F.J., et al., *Differential regulation of gonadotropin secretion by testosterone in the human male: absence of a negative feedback effect of testosterone on follicle-stimulating hormone secretion.* J Clin Endocrinol Metab, 2001. **86**(1): p. 53-8.
82. Matsumoto, A.M. and W.J. Bremner, *Modulation of pulsatile gonadotropin secretion by testosterone in man.* J Clin Endocrinol Metab, 1984. **58**(4): p. 609-14.
83. Walker, W.H. and J. Cheng, *FSH and testosterone signaling in Sertoli cells.* Reproduction, 2005. **130**(1): p. 15-28.
84. Petersen, C. and O. Soder, *The sertoli cell--a hormonal target and 'super' nurse for germ cells that determines testicular size.* Horm Res, 2006. **66**(4): p. 153-61.

85. Galdieri, M., L. Monaco, and M. Stefanini, *Secretion of androgen binding protein by Sertoli cells is influenced by contact with germ cells.* J Androl, 1984. **5**(6): p. 409-15.
86. Medhamurthy, R., et al., *Evidence that inhibin plays a major role in the regulation of follicle-stimulating hormone secretion in the fully adult male rhesus monkey (Macaca mulatta).* Endocrinology, 1991. **129**(1): p. 389-95.
87. Boepple, P.A., et al., *Relative roles of inhibin B and sex steroids in the negative feedback regulation of follicle-stimulating hormone in men across the full spectrum of seminiferous epithelium function.* J Clin Endocrinol Metab, 2008. **93**(5): p. 1809-14.
88. Jakubowiak, A., A. Janecki, and A. Steinberger, *Kinetics of inhibin secretion in static and superfused Sertoli cell cultures in response to follicle-stimulating hormone.* Biol Reprod, 1990. **43**(6): p. 939-45.
89. Fiddes, J.C. and K. Talmadge, *Structure, expression, and evolution of the genes for the human glycoprotein hormones.* Recent Prog Horm Res, 1984. **40**: p. 43-78.
90. Mercer, J.E. and W.W. Chin, *Regulation of pituitary gonadotrophin gene expression.* Hum Reprod Update, 1995. **1**(4): p. 363-84.
91. Ulloa-Aguirre, A. and C. Timossi, *Structure-function relationship of follicle-stimulating hormone and its receptor.* Hum Reprod Update, 1998. **4**(3): p. 260-83.
92. Maurer, R.A. and A. Beck, *Isolation and nucleotide sequence analysis of a cloned cDNA encoding the beta-subunit of bovine follicle-stimulating hormone.* DNA, 1986. **5**(5): p. 363-9.
93. Watkins, P.C., et al., *DNA sequence and regional assignment of the human follicle-stimulating hormone beta-subunit gene to the short arm of human chromosome 11.* DNA, 1987. **6**(3): p. 205-12.
94. Gharib, S.D., et al., *Isolation and characterization of the gene encoding the beta-subunit of rat follicle-stimulating hormone.* DNA, 1989. **8**(5): p. 339-49.
95. Lapthorn, A.J., et al., *Crystal structure of human chorionic gonadotropin.* Nature, 1994. **369**(6480): p. 455-61.
96. Zeng, H., I. Ji, and T.H. Ji, *Lys91 and His90 of the alpha-subunit are crucial for receptor binding and hormone action of follicle-stimulating hormone (FSH) and play hormone-specific roles in FSH and human chorionic gonadotropin.* Endocrinology, 1995. **136**(7): p. 2948-53.
97. Dias, J.A., *Human follitropin heterodimerization and receptor binding structural motifs: identification and analysis by a combination of synthetic peptide and mutagenesis approaches.* Mol Cell Endocrinol, 1996. **125**(1-2): p. 45-54.
98. Liu, C. and J.A. Dias, *Long loop residues 33-58 in the human glycoprotein hormone common alpha subunit contain structural components for subunit heterodimerization and human follitropin-receptor binding.* Arch Biochem Biophys, 1996. **329**(1): p. 127-35.
99. Fan, Q.R. and W.A. Hendrickson, *Structure of human follicle-stimulating hormone in complex with its receptor.* Nature, 2005. **433**(7023): p. 269-77.
100. Reichert, L.E., Jr. and B. Dattatreyamurty, *The follicle-stimulating hormone (FSH) receptor in testis: interaction with FSH, mechanism of signal transduction, and properties of the purified receptor.* Biol Reprod, 1989. **40**(1): p. 13-26.
101. DeManno, D.A., et al., *Follicle-stimulating hormone promotes histone H3 phosphorylation on serine-10.* Mol Endocrinol, 1999. **13**(1): p. 91-105.
102. Cottom, J., et al., *Follicle-stimulating hormone activates extracellular signal-regulated kinase but not extracellular signal-regulated kinase kinase through a 100-kDa phosphotyrosine phosphatase.* J Biol Chem, 2003. **278**(9): p. 7167-79.
103. Salvador, L.M., et al., *Follicle-stimulating hormone stimulates protein kinase A-mediated histone H3 phosphorylation and acetylation leading to select gene activation in ovarian granulosa cells.* J Biol Chem, 2001. **276**(43): p. 40146-55.
104. Ongeri, E.M., M.F. Verderame, and J.M. Hammond, *Follicle-stimulating hormone induction of ovarian insulin-like growth factor-binding protein-3 transcription requires a TATA box-*

binding protein and the protein kinase A and phosphatidylinositol-3 kinase pathways. Mol Endocrinol, 2005. **19**(7): p. 1837-48.
105. Maizels, E.T., et al., *Follicle stimulating hormone (FSH) activates the p38 mitogen-activated protein kinase pathway, inducing small heat shock protein phosphorylation and cell rounding in immature rat ovarian granulosa cells.* Endocrinology, 1998. **139**(7): p. 3353-6.
106. Yu, F.Q., et al., *Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially.* J Endocrinol, 2005. **186**(1): p. 85-96.
107. Sasson, R., et al., *Novel genes modulated by FSH in normal and immortalized FSH-responsive cells: new insights into the mechanism of FSH action.* FASEB J, 2003. **17**(10): p. 1256-66.
108. Grieshaber, N.A., et al., *Follicle-stimulating hormone-responsive cytoskeletal genes in rat granulosa cells: class I beta-tubulin, tropomyosin-4, and kinesin heavy chain.* Endocrinology, 2003. **144**(1): p. 29-39.
109. Tanaka, M., et al., *The generation and characterization of an ovary-selective cDNA library.* Mol Cell Endocrinol, 2003. **202**(1-2): p. 67-9.
110. Zeleznik, A.J., A.R. Midgley, Jr., and L.E. Reichert, Jr., *Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo.* Endocrinology, 1974. **95**(3): p. 818-25.
111. Sicinski, P., et al., *Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis.* Nature, 1996. **384**(6608): p. 470-4.
112. Orly, J., G. Sato, and G.F. Erickson, *Serum suppresses the expression of hormonally induced functions in cultured granulosa cells.* Cell, 1980. **20**(3): p. 817-27.
113. Walker, W.H., L. Fucci, and J.F. Habener, *Expression of the gene encoding transcription factor cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein (CREB): regulation by follicle-stimulating hormone-induced cAMP signaling in primary rat Sertoli cells.* Endocrinology, 1995. **136**(8): p. 3534-45.
114. Kumar, T.R., et al., *Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.* Nat Genet, 1997. **15**(2): p. 201-4.
115. Burns, K.H., et al., *Analysis of ovarian gene expression in follicle-stimulating hormone beta knockout mice.* Endocrinology, 2001. **142**(7): p. 2742-51.
116. Dierich, A., et al., *Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13612-7.
117. Krishnamurthy, H., et al., *Delay in sexual maturity of the follicle-stimulating hormone receptor knockout male mouse.* Biol Reprod, 2001. **65**(2): p. 522-31.
118. Kumar, T.R., et al., *Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis.* Mol Endocrinol, 1999. **13**(6): p. 851-65.
119. Matthews, C.H., et al., *Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone.* Nat Genet, 1993. **5**(1): p. 83-6.
120. Layman, L.C., et al., *Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene.* N Engl J Med, 1997. **337**(9): p. 607-11.
121. Kottler, M.L., et al., *FSHbeta gene mutation in a female with delayed puberty and hypogonadism: response to recombinant human FSH.* Folia Histochem Cytobiol, 2009. **47**(5): p. S55-8.
122. Lindstedt, G., et al., *Follitropin (FSH) deficiency in an infertile male due to FSHbeta gene mutation. A syndrome of normal puberty and virilization but underdeveloped testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations.* Clin Chem Lab Med, 1998. **36**(8): p. 663-5.

123. Phillip, M., et al., *Male hypogonadism due to a mutation in the gene for the beta-subunit of follicle-stimulating hormone.* N Engl J Med, 1998. **338**(24): p. 1729-32.
124. Clark, A.D. and L.C. Layman, *Analysis of the Cys82Arg mutation in follicle-stimulating hormone beta (FSHbeta) using a novel FSH expression vector.* Fertil Steril, 2003. **79**(2): p. 379-85.
125. Maroulis, G.B., A.F. Parlow, and J.R. Marshall, *Isolated follicle-stimulating hormone deficiency in man.* Fertil Steril, 1977. **28**(8): p. 818-22.
126. Hagg, E., C. Tollin, and B. Bergman, *Isolated FSH deficiency in a male. A case report.* Scand J Urol Nephrol, 1978. **12**(3): p. 287-9.
127. Aittomaki, K., et al., *Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure.* Cell, 1995. **82**(6): p. 959-68.
128. Aittomaki, K., et al., *Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene.* J Clin Endocrinol Metab, 1996. **81**(10): p. 3722-6.
129. Beau, I., et al., *A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor.* J Clin Invest, 1998. **102**(7): p. 1352-9.
130. Tapanainen, J.S., et al., *Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility.* Nat Genet, 1997. **15**(2): p. 205-6.
131. Huhtaniemi, I.T. and K. Aittomaki, *Mutations of follicle-stimulating hormone and its receptor: effects on gonadal function.* Eur J Endocrinol, 1998. **138**(5): p. 473-81.
132. Lathi, R.B. and A.A. Milki, *Recombinant gonadotropins.* Curr Womens Health Rep, 2001. **1**(2): p. 157-63.
133. Lunenfeld, B., *Development of gonadotrophins for clinical use.* Reprod Biomed Online, 2002. **4 Suppl 1**: p. 11-7.
134. Weinbauer, G.F., et al., *Human follicle-stimulating hormone exerts a stimulatory effect on spermatogenesis, testicular size, and serum inhibin levels in the gonadotropin-releasing hormone antagonist-treated nonhuman primate (Macaca fascicularis).* Endocrinology, 1991. **129**(4): p. 1831-9.
135. Gemzell, C.A., E. Diczfalusy, and G. Tillinger, *Clinical effect of human pituitary follicle-stimulating hormone (FSH).* J Clin Endocrinol Metab, 1958. **18**(12): p. 1333-48.
136. Rosemberg, E., et al., *Clinical Effect of Gonadotropins of Human Origin. Case Report with a Two-Year Follow-Up.* J Clin Endocrinol Metab, 1964. **24**: p. 105-17.
137. Gindoff, P.R. and R. Jewelewicz, *Progress in ovulation induction with gonadotropins.* Bull N Y Acad Med, 1989. **65**(5): p. 574-82.
138. Polan, M.L., et al., *Ovulation induction with human menopausal gonadotropin compared to human urinary follicle-stimulating hormone results in a significant shift in follicular fluid androgen levels without discernible differences in granulosa-luteal cell function.* J Clin Endocrinol Metab, 1986. **63**(6): p. 1284-91.
139. Gleicher, N., M. Vietzke, and A. Vidali, *Bye-bye urinary gonadotrophins? Recombinant FSH: a real progress in ovulation induction and IVF?* Hum Reprod, 2003. **18**(3): p. 476-82.
140. *IVF- In vitro Fertilization*. Available from: http://www.fertilityconnect.com/ivf.html.
141. *In Vitro Fertilization*. Available from: http://www.emedicinehealth.com/in_vitro_fertilization/article_em.htm.
142. *IVF Treatment Sequence Timeline*. Available from: http://www.kpivf.com/sequence.cfm.
143. Foresta, C., et al., *Follicle-stimulating hormone treatment of male infertility.* Curr Opin Urol, 2008. **18**(6): p. 602-7.
144. Foresta, C., et al., *Recombinant FSH in the treatment of oligozoospermia.* Expert Opin Biol Ther, 2009. **9**(5): p. 659-66.

145. Foresta, C., et al., *Treatment of male idiopathic infertility with recombinant human follicle-stimulating hormone: a prospective, controlled, randomized clinical study.* Fertil Steril, 2005. **84**(3): p. 654-61.
146. Foresta, C., et al., *Hormonal treatment of male infertility: FSH.* Reprod Biomed Online, 2007. **15**(6): p. 666-72.
147. Lee, D.W., et al., *A decapeptide corresponding to the partial amino acid sequence of a high molecular weight human FSH receptor-binding inhibitor is a specific inhibitor of FSH binding.* Pept Res, 1995. **8**(3): p. 171-7.
148. Dattatreyamurty, B. and L.E. Reichert, Jr., *A synthetic peptide corresponding to amino acids 9-30 of the extracellular domain of the follitropin (FSH) receptor specifically binds FSH.* Mol Cell Endocrinol, 1992. **87**(1-3): p. 9-17.
149. Santa Coloma, T.A., B. Dattatreyamurty, and L.E. Reichert, Jr., *A synthetic peptide corresponding to human FSH beta-subunit 33-53 binds to FSH receptor, stimulates basal estradiol biosynthesis, and is a partial antagonist of FSH.* Biochemistry, 1990. **29**(5): p. 1194-200.
150. van Straten, N.C., et al., *Identification of substituted 6-amino-4-phenyltetrahydroquinoline derivatives: potent antagonists for the follicle-stimulating hormone receptor.* J Med Chem, 2005. **48**(6): p. 1697-700.
151. Arey, B.J., et al., *Identification and characterization of a selective, nonpeptide follicle-stimulating hormone receptor antagonist.* Endocrinology, 2002. **143**(10): p. 3822-9.
152. Abdennebi, L., et al., *Generating FSH antagonists and agonists through immunization against FSH receptor N-terminal decapeptides.* J Mol Endocrinol, 1999. **22**(2): p. 151-9.
153. Moudgal, N.R., et al., *Long-term contraceptive efficacy of vaccine of ovine follicle-stimulating hormone in male bonnet monkeys (Macaca radiata).* J Reprod Fertil, 1992. **96**(1): p. 91-102.
154. Moudgal, N.R., et al., *Development of male contraceptive vaccine--a perspective.* Hum Reprod Update, 1997. **3**(4): p. 335-46.
155. Sunyer, T., et al., *Estrogen's bone-protective effects may involve differential IL-1 receptor regulation in human osteoclast-like cells.* J Clin Invest, 1999. **103**(10): p. 1409-18.
156. Kameda, T., et al., *Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts.* J Exp Med, 1997. **186**(4): p. 489-95.
157. Cosman, F., et al., *Estrogen protection against bone resorbing effects of parathyroid hormone infusion. Assessment by use of biochemical markers.* Ann Intern Med, 1993. **118**(5): p. 337-43.
158. Grassi, F., et al., *Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation.* Proc Natl Acad Sci U S A, 2007. **104**(38): p. 15087-92.
159. Gross, M.L., et al., *Effects of estrogens on cardiovascular structure in uninephrectomized SHRsp rats.* Kidney Int, 2005. **67**(3): p. 849-57.
160. Kim, J.K. and E.R. Levin, *Estrogen signaling in the cardiovascular system.* Nucl Recept Signal, 2006. **4**: p. e013.
161. Otsuki, M., K. Dahlman-Wright, and J.A. Gustafsson, *Cardiovascular roles of estrogen receptors: insights gained from knockout models.* Nucl Recept Signal, 2003. **1**: p. e003.
162. Mendelsohn, M.E. and R.H. Karas, *The protective effects of estrogen on the cardiovascular system.* N Engl J Med, 1999. **340**(23): p. 1801-11.
163. Stampfer, M.J., et al., *Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the nurses' health study.* N Engl J Med, 1991. **325**(11): p. 756-62.
164. Schwartz, J., R. Freeman, and W. Frishman, *Clinical pharmacology of estrogens: cardiovascular actions and cardioprotective benefits of replacement therapy in postmenopausal women.* J Clin Pharmacol, 1995. **35**(3): p. 314-29.

165. Nicol, L., et al., *Differential secretion of gonadotrophins: investigation of the role of secretogranin II and chromogranin A in the release of LH and FSH in LbetaT2 cells.* J Mol Endocrinol, 2004. **32**(2): p. 467-80.
166. Farnworth, P.G., *Gonadotrophin secretion revisited. How many ways can FSH leave a gonadotroph?* J Endocrinol, 1995. **145**(3): p. 387-95.
167. McNeilly, A.S., et al., *The differential secretion of FSH and LH: regulation through genes, feedback and packaging.* Reprod Suppl, 2003. **61**: p. 463-76.
168. Alarid, E.T., et al., *Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice.* Development, 1996. **122**(10): p. 3319-29.
169. Graham, K.E., K.D. Nusser, and M.J. Low, *LbetaT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to active A.* J Endocrinol, 1999. **162**(3): p. R1-5.
170. Pernasetti, F., et al., *Cell-specific transcriptional regulation of follicle-stimulating hormone-beta by activin and gonadotropin-releasing hormone in the LbetaT2 pituitary gonadotrope cell model.* Endocrinology, 2001. **142**(6): p. 2284-95.
171. Wu, J.C., et al., *Rapid, efficient isolation of murine gonadotropes and their use in revealing control of follicle-stimulating hormone by paracrine pituitary factors.* Endocrinology, 2004. **145**(12): p. 5832-9.
172. Strahl, B.D., et al., *Transcriptional activation of the ovine follicle-stimulating hormone beta-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C.* Endocrinology, 1998. **139**(11): p. 4455-65.
173. Lin, S.W. and W. Ge, *Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish--an in vitro approach.* Gen Comp Endocrinol, 2009. **160**(2): p. 183-93.
174. Coss, D., et al., *Activin regulates luteinizing hormone beta-subunit gene expression through Smad-binding and homeobox elements.* Mol Endocrinol, 2005. **19**(10): p. 2610-23.
175. Lau, M.T. and W. Ge, *Cloning of Smad2, Smad3, Smad4, and Smad7 from the goldfish pituitary and evidence for their involvement in activin regulation of goldfish FSHbeta promoter activity.* Gen Comp Endocrinol, 2005. **141**(1): p. 22-38.
176. Ando, H., et al., *Synergistic effects of salmon gonadotropin-releasing hormone and estradiol-17beta on gonadotropin subunit gene expression and release in masu salmon pituitary cells in vitro.* Gen Comp Endocrinol, 2004. **137**(1): p. 109-21.
177. Bohnsack, B.L., et al., *Follistatin suppresses steroid-enhanced follicle-stimulating hormone release in vitro in rats.* Biol Reprod, 2000. **62**(3): p. 636-41.
178. Melamed, P. and Z. Yaron, *Calcium ionophores lead to apoptotic-like changes in Tilapia pituitary cells.* Gen Comp Endocrinol, 1999. **114**(1): p. 19-27.
179. Huang, Y.S., et al., *Cortisol selectively stimulates pituitary gonadotropin beta-subunit in a primitive teleost, Anguilla anguilla.* Endocrinology, 1999. **140**(3): p. 1228-35.
180. Whitfield, G.K. and W.L. Miller, *Biosynthesis and secretion of follicle-stimulating hormone beta-subunit from ovine pituitary cultures: effect of 17 beta-estradiol treatment.* Endocrinology, 1984. **115**(1): p. 154-9.
181. Ooi, G.T., N. Tawadros, and R.M. Escalona, *Pituitary cell lines and their endocrine applications.* Mol Cell Endocrinol, 2004. **228**(1-2): p. 1-21.
182. Childs, G.V. and G. Unabia, *The use of counterflow centrifugation to enrich gonadotropes and somatotropes.* J Histochem Cytochem, 2001. **49**(5): p. 663-4.
183. Stanislaus, D., et al., *Regulation of G(q/11)alpha by the gonadotropin-releasing hormone receptor.* Mol Endocrinol, 1997. **11**(6): p. 738-46.
184. Haisenleder, D.J., H.A. Ferris, and M.A. Shupnik, *The calcium component of gonadotropin-releasing hormone-stimulated luteinizing hormone subunit gene transcription is mediated by calcium/calmodulin-dependent protein kinase type II.* Endocrinology, 2003. **144**(6): p. 2409-16.

185. Liu, F., et al., *GnRH activates ERK1/2 leading to the induction of c-fos and LHbeta protein expression in LbetaT2 cells.* Mol Endocrinol, 2002. **16**(3): p. 419-34.
186. Roberson, M.S., et al., *A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone alpha-subunit promoter by gonadotropin-releasing hormone.* Mol Cell Biol, 1995. **15**(7): p. 3531-9.
187. Sundaresan, S., et al., *Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone: evidence for the involvement of protein kinase C.* Endocrinology, 1996. **137**(1): p. 304-11.
188. Bonfil, D., et al., *Extracellular signal-regulated kinase, Jun N-terminal kinase, p38, and c-Src are involved in gonadotropin-releasing hormone-stimulated activity of the glycoprotein hormone follicle-stimulating hormone beta-subunit promoter.* Endocrinology, 2004. **145**(5): p. 2228-44.
189. Vasilyev, V.V., et al., *Transcriptional activation of the ovine follicle-stimulating hormone-beta gene by gonadotropin-releasing hormone involves multiple signal transduction pathways.* Endocrinology, 2002. **143**(5): p. 1651-9.
190. Kakar, S.S., et al., *Identification of distinct gene expression profiles associated with treatment of LbetaT2 cells with gonadotropin-releasing hormone agonist using microarray analysis.* Gene, 2003. **308**: p. 67-77.
191. Wurmbach, E., et al., *Gonadotropin-releasing hormone receptor-coupled gene network organization.* J Biol Chem, 2001. **276**(50): p. 47195-201.
192. Coss, D., et al., *p38 mitogen-activated protein kinase is critical for synergistic induction of the FSH(beta) gene by gonadotropin-releasing hormone and activin through augmentation of c-Fos induction and Smad phosphorylation.* Mol Endocrinol, 2007. **21**(12): p. 3071-86.
193. Wang, Y., et al., *Activator protein-1 and smad proteins synergistically regulate human follicle-stimulating hormone beta-promoter activity.* Endocrinology, 2008. **149**(11): p. 5577-91.
194. Bliss, S.P., et al., *ERK signaling in the pituitary is required for female but not male fertility.* Mol Endocrinol, 2009. **23**(7): p. 1092-101.
195. Strahl, B.D., et al., *Two proximal activating protein-1-binding sites are sufficient to stimulate transcription of the ovine follicle-stimulating hormone-beta gene.* Endocrinology, 1997. **138**(6): p. 2621-31.
196. Coss, D., et al., *A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone beta gene by gonadotropin-releasing hormone.* J Biol Chem, 2004. **279**(1): p. 152-62.
197. Huang, H.J., et al., *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, 2001. **142**(6): p. 2260-6.
198. Ciccone, N.A., et al., *A composite element that binds basic helix loop helix and basic leucine zipper transcription factors is important for gonadotropin-releasing hormone regulation of the follicle-stimulating hormone beta gene.* Mol Endocrinol, 2008. **22**(8): p. 1908-23.
199. Burger, L.L., et al., *Regulation of gonadotropin subunit gene transcription.* J Mol Endocrinol, 2004. **33**(3): p. 559-84.
200. Thackray, V.G., S.M. McGillivray, and P.L. Mellon, *Androgens, progestins, and glucocorticoids induce follicle-stimulating hormone beta-subunit gene expression at the level of the gonadotrope.* Mol Endocrinol, 2006. **20**(9): p. 2062-79.
201. Webster, J.C., et al., *The 5'-flanking region of the ovine follicle-stimulating hormone-beta gene contains six progesterone response elements: three proximal elements are sufficient to increase transcription in the presence of progesterone.* Endocrinology, 1995. **136**(3): p. 1049-58.
202. Batra, S.K. and W.L. Miller, *Progesterone inhibits basal production of follicle-stimulating hormone in ovine pituitary cell culture.* Endocrinology, 1985. **117**(6): p. 2443-8.

203. Thackray, V.G. and P.L. Mellon, *Synergistic induction of follicle-stimulating hormone beta-subunit gene expression by gonadal steroid hormone receptors and Smad proteins.* Endocrinology, 2008. **149**(3): p. 1091-102.
204. Kumar, T.R. and M.J. Low, *Hormonal regulation of human follicle-stimulating hormone-beta subunit gene expression: GnRH stimulation and GnRH-independent androgen inhibition.* Neuroendocrinology, 1995. **61**(6): p. 628-37.
205. Finkelstein, J.S., et al., *Sex steroid control of gonadotropin secretion in the human male. I. Effects of testosterone administration in normal and gonadotropin-releasing hormone-deficient men.* J Clin Endocrinol Metab, 1991. **73**(3): p. 609-20.
206. Sheckter, C.B., A.M. Matsumoto, and W.J. Bremner, *Testosterone administration inhibits gonadotropin secretion by an effect directly on the human pituitary.* J Clin Endocrinol Metab, 1989. **68**(2): p. 397-401.
207. Winters, S.J., *FSH is produced by GnRH-deficient men and is suppressed by testosterone.* J Androl, 1994. **15**(3): p. 216-9.
208. Shupnik, M.A., S.D. Gharib, and W.W. Chin, *Estrogen suppresses rat gonadotropin gene transcription in vivo.* Endocrinology, 1988. **122**(5): p. 1842-6.
209. Gieske, M.C., et al., *Pituitary gonadotroph estrogen receptor-alpha is necessary for fertility in females.* Endocrinology, 2008. **149**(1): p. 20-7.
210. Glidewell-Kenney, C., et al., *Estrogen receptor alpha signaling pathways differentially regulate gonadotropin subunit gene expression and serum follicle-stimulating hormone in the female mouse.* Endocrinology, 2008. **149**(8): p. 4168-76.
211. Dalkin, A.C., et al., *Gonadal regulation of gonadotropin subunit gene expression: evidence for regulation of follicle-stimulating hormone-beta messenger ribonucleic acid by nonsteroidal hormones in female rats.* Endocrinology, 1990. **127**(2): p. 798-806.
212. Dalkin, A.C., et al., *Ovariectomy and inhibin immunoneutralization acutely increase follicle-stimulating hormone-beta messenger ribonucleic acid concentrations: evidence for a nontranscriptional mechanism.* Endocrinology, 1993. **132**(3): p. 1297-304.
213. Gharib, S.D., et al., *Sex steroid hormone regulation of follicle-stimulating hormone subunit messenger ribonucleic acid (mRNA) levels in the rat.* J Clin Invest, 1987. **80**(2): p. 294-9.
214. Melamed, P., et al., *Transcription of gonadotropin beta subunit genes involves cross-talk between the transcription factors and co-regulators that mediate actions of the regulatory hormones.* Mol Cell Endocrinol, 2006. **252**(1-2): p. 167-83.
215. Attardi, B. and T. Fitzgerald, *Effects of progesterone on the estradiol-induced follicle-stimulating hormone (FSH) surge and FSH beta messenger ribonucleic acid in the rat.* Endocrinology, 1990. **126**(5): p. 2281-7.
216. Tebar, M., F.H. de Jong, and J.E. Sanchez-Criado, *Regulation of inhibin/activin subunits and follistatin mRNA expression in the rat pituitary at early estrus.* Life Sci, 2000. **67**(21): p. 2549-62.
217. Ringstrom, S.J., et al., *The antiprogestins RU486 and ZK98299 affect follicle-stimulating hormone secretion differentially on estrus, but not on proestrus.* Endocrinology, 1997. **138**(6): p. 2286-90.
218. Szabo, M., et al., *Antiprogestins suppress basal and activin-stimulated follicle-stimulating hormone secretion in an estrogen-dependent manner.* Endocrinology, 1998. **139**(5): p. 2223-8.
219. O'Conner, J.L., et al., *A 361 base pair region of the rat FSH-beta promoter contains multiple progesterone receptor-binding sequences and confers progesterone responsiveness.* Mol Cell Endocrinol, 1997. **136**(1): p. 67-78.
220. Phillips, C.L., et al., *17 Beta-estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle-stimulating hormone.* Mol Endocrinol, 1988. **2**(7): p. 641-9.

221. Miyake, T., et al., *Interaction of activin A and gonadal steroids on FSH secretion from primary cultured rat anterior pituitary cells.* Biochem Biophys Res Commun, 1993. **194**(1): p. 413-9.
222. Bernard, D.J., et al., *Mechanisms of FSH synthesis: what we know, what we don't, and why you should care.* Fertil Steril. **93**(8): p. 2465-85.
223. Derynck, R. and K. Miyazono, eds. *The TGF-β Family*. 2008, Cold Spring Harbor Laboratory Press: Cold Spring Harbor. p. 121-139.
224. Ling, N., et al., *Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid.* Proc Natl Acad Sci U S A, 1985. **82**(21): p. 7217-21.
225. Miyamoto, K., et al., *Isolation of porcine follicular fluid inhibin of 32K daltons.* Biochem Biophys Res Commun, 1985. **129**(2): p. 396-403.
226. Rivier, J., et al., *Purification and partial characterization of inhibin from porcine follicular fluid.* Biochem Biophys Res Commun, 1985. **133**(1): p. 120-7.
227. Robertson, D.M., et al., *Isolation of inhibin from bovine follicular fluid.* Biochem Biophys Res Commun, 1985. **126**(1): p. 220-6.
228. Miyazono, K., Y. Kamiya, and M. Morikawa, *Bone morphogenetic protein receptors and signal transduction.* J Biochem, 2010. **147**(1): p. 35-51.
229. Chen, Y.G., *Endocytic regulation of TGF-beta signaling.* Cell Res, 2009. **19**(1): p. 58-70.
230. Hartung, A., et al., *Different routes of bone morphogenic protein (BMP) receptor endocytosis influence BMP signaling.* Mol Cell Biol, 2006. **26**(20): p. 7791-805.
231. Hayes, S., A. Chawla, and S. Corvera, *TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2.* J Cell Biol, 2002. **158**(7): p. 1239-49.
232. Tsukazaki, T., et al., *SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor.* Cell, 1998. **95**(6): p. 779-91.
233. Di Guglielmo, G.M., et al., *Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover.* Nat Cell Biol, 2003. **5**(5): p. 410-21.
234. Lin, H.K., S. Bergmann, and P.P. Pandolfi, *Cytoplasmic PML function in TGF-beta signalling.* Nature, 2004. **431**(7005): p. 205-11.
235. Miura, S., et al., *Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA.* Mol Cell Biol, 2000. **20**(24): p. 9346-55.
236. Goumans, M.J., et al., *Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling.* Mol Cell, 2003. **12**(4): p. 817-28.
237. Lebrin, F., et al., *Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction.* EMBO J, 2004. **23**(20): p. 4018-28.
238. Daly, A.C., R.A. Randall, and C.S. Hill, *Transforming growth factor beta-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth.* Mol Cell Biol, 2008. **28**(22): p. 6889-902.
239. Murakami, M., et al., *Receptor expression modulates the specificity of transforming growth factor-beta signaling pathways.* Genes Cells, 2009. **14**(4): p. 469-82.
240. Sieber, C., et al., *Recent advances in BMP receptor signaling.* Cytokine Growth Factor Rev, 2009. **20**(5-6): p. 343-55.
241. Shi, Y., et al., *Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling.* Cell, 1998. **94**(5): p. 585-94.
242. Huse, M., et al., *Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12.* Cell, 1999. **96**(3): p. 425-36.
243. Wu, G., et al., *Structural basis of Smad2 recognition by the Smad anchor for receptor activation.* Science, 2000. **287**(5450): p. 92-7.
244. Feng, X.H. and R. Derynck, *A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity.* EMBO J, 1997. **16**(13): p. 3912-23.

245. Chen, Y.G., et al., *Determinants of specificity in TGF-beta signal transduction.* Genes Dev, 1998. **12**(14): p. 2144-52.
246. Persson, U., et al., *The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation.* FEBS Lett, 1998. **434**(1-2): p. 83-7.
247. Pierreux, C.E., F.J. Nicolas, and C.S. Hill, *Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus.* Mol Cell Biol, 2000. **20**(23): p. 9041-54.
248. Watanabe, M., et al., *Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal.* EMBO Rep, 2000. **1**(2): p. 176-82.
249. Inman, G.J., F.J. Nicolas, and C.S. Hill, *Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity.* Mol Cell, 2002. **10**(2): p. 283-94.
250. Massague, J., J. Seoane, and D. Wotton, *Smad transcription factors.* Genes Dev, 2005. **19**(23): p. 2783-810.
251. Xu, L., et al., *Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus.* Mol Cell, 2002. **10**(2): p. 271-82.
252. Wu, J.W., et al., *Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling.* Mol Cell, 2001. **8**(6): p. 1277-89.
253. Xu, L. and J. Massague, *Nucleocytoplasmic shuttling of signal transducers.* Nat Rev Mol Cell Biol, 2004. **5**(3): p. 209-19.
254. Shi, Y. and J. Massague, *Mechanisms of TGF-beta signaling from cell membrane to the nucleus.* Cell, 2003. **113**(6): p. 685-700.
255. Shi, Y., et al., *A structural basis for mutational inactivation of the tumour suppressor Smad4.* Nature, 1997. **388**(6637): p. 87-93.
256. Gao, S., et al., *Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling.* Mol Cell, 2009. **36**(3): p. 457-68.
257. Hayashi, H., et al., *The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling.* Cell, 1997. **89**(7): p. 1165-73.
258. Nakao, A., et al., *Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling.* Nature, 1997. **389**(6651): p. 631-5.
259. Imamura, T., et al., *Smad6 inhibits signalling by the TGF-beta superfamily.* Nature, 1997. **389**(6651): p. 622-6.
260. Hata, A., et al., *Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor.* Genes Dev, 1998. **12**(2): p. 186-97.
261. Goto, K., et al., *Selective inhibitory effects of Smad6 on bone morphogenetic protein type I receptors.* J Biol Chem, 2007. **282**(28): p. 20603-11.
262. Lin, X., et al., *Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription.* Mol Cell Biol, 2003. **23**(24): p. 9081-93.
263. Zhang, S., et al., *Smad7 antagonizes transforming growth factor beta signaling in the nucleus by interfering with functional Smad-DNA complex formation.* Mol Cell Biol, 2007. **27**(12): p. 4488-99.
264. Kavsak, P., et al., *Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation.* Mol Cell, 2000. **6**(6): p. 1365-75.
265. Suzuki, C., et al., *Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane.* J Biol Chem, 2002. **277**(42): p. 39919-25.
266. Tajima, Y., et al., *Chromosomal region maintenance 1 (CRM1)-dependent nuclear export of Smad ubiquitin regulatory factor 1 (Smurf1) is essential for negative regulation of transforming growth factor-beta signaling by Smad7.* J Biol Chem, 2003. **278**(12): p. 10716-21.

267. Ishisaki, A., et al., *Smad7 is an activin-inducible inhibitor of activin-induced growth arrest and apoptosis in mouse B cells.* J Biol Chem, 1998. **273**(38): p. 24293-6.
268. Afrakhte, M., et al., *Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members.* Biochem Biophys Res Commun, 1998. **249**(2): p. 505-11.
269. Ishida, W., et al., *Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter.* J Biol Chem, 2000. **275**(9): p. 6075-9.
270. Vale, W., et al., *Chemical and biological characterization of the inhibin family of protein hormones.* Recent Prog Horm Res, 1988. **44**: p. 1-34.
271. Ying, S.Y., *Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone.* Endocr Rev, 1988. **9**(2): p. 267-93.
272. Bilezikjian, L.M., et al., *Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin.* Mol Cell Endocrinol, 2004. **225**(1-2): p. 29-36.
273. Ling, N., et al., *Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin.* Nature, 1986. **321**(6072): p. 779-82.
274. Vale, W., et al., *Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid.* Nature, 1986. **321**(6072): p. 776-9.
275. G, H.t., et al., *Cloning of a new member of the TGF-beta family: a putative new activin beta C chain.* Biochem Biophys Res Commun, 1995. **206**(2): p. 608-13.
276. Oda, S., et al., *Molecular cloning and functional analysis of a new activin beta subunit: a dorsal mesoderm-inducing activity in Xenopus.* Biochem Biophys Res Commun, 1995. **210**(2): p. 581-8.
277. Fang, J., et al., *Molecular cloning of the mouse activin beta E subunit gene.* Biochem Biophys Res Commun, 1996. **228**(3): p. 669-74.
278. Mellor, S.L., et al., *Localization of activin beta(A)-, beta(B)-, and beta(C)-subunits in humanprostate and evidence for formation of new activin heterodimers of beta(C)-subunit.* J Clin Endocrinol Metab, 2000. **85**(12): p. 4851-8.
279. Michel, U., P. Farnworth, and J.K. Findlay, *Follistatins: more than follicle-stimulating hormone suppressing proteins.* Mol Cell Endocrinol, 1993. **91**(1-2): p. 1-11.
280. Phillips, D.J. and D.M. de Kretser, *Follistatin: a multifunctional regulatory protein.* Front Neuroendocrinol, 1998. **19**(4): p. 287-322.
281. McConnell, D.S., et al., *A two-site chemiluminescent assay for activin-free follistatin reveals that most follistatin circulating in men and normal cycling women is in an activin-bound state.* J Clin Endocrinol Metab, 1998. **83**(3): p. 851-8.
282. Lee, K.B., et al., *Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone beta subunit transcription.* J Mol Endocrinol, 2007. **38**(1-2): p. 315-30.
283. Roberts, V., et al., *Production and regulation of inhibin subunits in pituitary gonadotropes.* Endocrinology, 1989. **124**(1): p. 552-4.
284. Corrigan, A.Z., et al., *Evidence for an autocrine role of activin B within rat anterior pituitary cultures.* Endocrinology, 1991. **128**(3): p. 1682-4.
285. DePaolo, L.V., L.N. Bald, and B.M. Fendly, *Passive immunoneutralization with a monoclonal antibody reveals a role for endogenous activin-B in mediating FSH hypersecretion during estrus and following ovariectomy of hypophysectomized, pituitary-grafted rats.* Endocrinology, 1992. **130**(3): p. 1741-3.
286. McGillivray, S.M., et al., *Activin and glucocorticoids synergistically activate follicle-stimulating hormone beta-subunit gene expression in the immortalized LbetaT2 gonadotrope cell line.* Endocrinology, 2007. **148**(2): p. 762-73.
287. Spady, T.J., et al., *Androgen regulates follicle-stimulating hormone beta gene expression in an activin-dependent manner in immortalized gonadotropes.* Mol Endocrinol, 2004. **18**(4): p. 925-40.

288. Vassalli, A., et al., *Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction.* Genes Dev, 1994. **8**(4): p. 414-27.
289. Matzuk, M.M., T.R. Kumar, and A. Bradley, *Different phenotypes for mice deficient in either activins or activin receptor type II.* Nature, 1995. **374**(6520): p. 356-60.
290. Kumar, T.R., et al., *Regulation of FSHbeta and GnRH receptor gene expression in activin receptor II knockout male mice.* Mol Cell Endocrinol, 2003. **212**(1-2): p. 19-27.
291. Dupont, J., et al., *Activin signaling pathways in ovine pituitary and LbetaT2 gonadotrope cells.* Biol Reprod, 2003. **68**(5): p. 1877-87.
292. Suszko, M.I. and T.K. Woodruff, *Cell-specificity of transforming growth factor-beta response is dictated by receptor bioavailability.* J Mol Endocrinol, 2006. **36**(3): p. 591-600.
293. Bernard, D.J., K.B. Lee, and M.M. Santos, *Activin B can signal through both ALK4 and ALK7 in gonadotrope cells.* Reprod Biol Endocrinol, 2006. **4**: p. 52.
294. Gu, Z., et al., *The type I activin receptor ActRIB is required for egg cylinder organization and gastrulation in the mouse.* Genes Dev, 1998. **12**(6): p. 844-57.
295. Jornvall, H., et al., *ALK7, a receptor for nodal, is dispensable for embryogenesis and left-right patterning in the mouse.* Mol Cell Biol, 2004. **24**(21): p. 9383-9.
296. Lamba, P., et al., *Acute regulation of murine follicle-stimulating hormone beta subunit transcription by activin A.* J Mol Endocrinol, 2006. **36**(1): p. 201-20.
297. Bernard, D.J., *Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells.* Mol Endocrinol, 2004. **18**(3): p. 606-23.
298. Bailey, J.S., et al., *Activin regulation of the follicle-stimulating hormone beta-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1.* Mol Endocrinol, 2004. **18**(5): p. 1158-70.
299. West, B.E., et al., *Regulation of the follicle-stimulating hormone beta gene by the LHX3 LIM-homeodomain transcription factor.* Endocrinology, 2004. **145**(11): p. 4866-79.
300. Suszko, M.I., et al., *Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin.* Mol Endocrinol, 2003. **17**(3): p. 318-32.
301. Suszko, M.I., et al., *Smad3 mediates activin-induced transcription of follicle-stimulating hormone beta-subunit gene.* Mol Endocrinol, 2005. **19**(7): p. 1849-58.
302. Gregory, S.J., et al., *Synergy between activin A and gonadotropin-releasing hormone in transcriptional activation of the rat follicle-stimulating hormone-beta gene.* Mol Endocrinol, 2005. **19**(1): p. 237-54.
303. Lamba, P., et al., *A novel role for the forkhead transcription factor FOXL2 in activin A-regulated follicle-stimulating hormone beta subunit transcription.* Mol Endocrinol, 2009. **23**(7): p. 1001-13.
304. Wang, Y., V. Libasci, and D.J. Bernard, *Activin A induction of FSHbeta subunit transcription requires SMAD4 in immortalized gonadotropes.* J Mol Endocrinol, 2010. **44**(6): p. 349-62.
305. Zawel, L., et al., *Human Smad3 and Smad4 are sequence-specific transcription activators.* Mol Cell, 1998. **1**(4): p. 611-7.
306. Su, P., et al., *Expression and regulation of the beta-subunit of ovine follicle-stimulating hormone relies heavily on a promoter sequence likely to bind Smad-associated proteins.* Endocrinology, 2007. **148**(9): p. 4500-8.
307. Lamba, P., et al., *Paired-like homeodomain transcription factors 1 and 2 regulate follicle-stimulating hormone beta-subunit transcription through a conserved cis-element.* Endocrinology, 2008. **149**(6): p. 3095-108.
308. Suszko, M.I., et al., *Smad3 and Pitx2 cooperate in stimulation of FSHbeta gene transcription.* Mol Cell Endocrinol, 2008. **281**(1-2): p. 27-36.
309. Zakaria, M.M., et al., *Pituitary homeobox 1 activates the rat FSHbeta (rFSHbeta) gene through both direct and indirect interactions with the rFSHbeta gene promoter.* Mol Endocrinol, 2002. **16**(8): p. 1840-52.

310. Han, S.O. and W.L. Miller, *Activin A induces ovine follicle stimulating hormone beta using -169/-58 bp of its promoter and a simple TATA box.* Reprod Biol Endocrinol, 2009. **7**: p. 66.
311. Blount, A.L., et al., *FoxL2 and Smad3 coordinately regulate follistatin gene transcription.* J Biol Chem, 2009. **284**(12): p. 7631-45.
312. Lamba, P., et al., *Activin A Regulates Porcine Follicle-Stimulating Hormone beta-Subunit Transcription via Cooperative Actions of SMADs and FOXL2.* Endocrinology, 2010. **151**(11): p. In press.
313. Corpuz, P.S., et al., *FoxL2 Is required for activin induction of the mouse and human follicle-stimulating hormone beta-subunit genes.* Mol Endocrinol, 2010. **24**(5): p. 1037-51.
314. Zhang, H., et al., *Activin modulates the transcriptional response of LbetaT2 cells to gonadotropin-releasing hormone and alters cellular proliferation.* Mol Endocrinol, 2006. **20**(11): p. 2909-30.
315. Safwat, N., et al., *Transforming growth factor beta-activated kinase 1 is a key mediator of ovine follicle-stimulating hormone beta-subunit expression.* Endocrinology, 2005. **146**(11): p. 4814-24.
316. Moriguchi, T., et al., *A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3.* J Biol Chem, 1996. **271**(23): p. 13675-9.
317. Yamaguchi, K., et al., *Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction.* Science, 1995. **270**(5244): p. 2008-11.
318. de Kretser, D.M., et al., *Inhibins, activins and follistatin in reproduction.* Hum Reprod Update, 2002. **8**(6): p. 529-41.
319. Welt, C., et al., *Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium.* Exp Biol Med (Maywood), 2002. **227**(9): p. 724-52.
320. Rivier, C. and W. Vale, *Immunoneutralization of endogenous inhibin modifies hormone secretion and ovulation rate in the rat.* Endocrinology, 1989. **125**(1): p. 152-7.
321. Attardi, B., J. Vaughan, and W. Vale, *Regulation of FSH beta messenger ribonucleic acid levels in the rat by endogenous inhibin.* Endocrinology, 1992. **130**(1): p. 557-9.
322. Attardi, B. and J. Miklos, *Rapid stimulatory effect of activin-A on messenger RNA encoding the follicle-stimulating hormone beta-subunit in rat pituitary cell cultures.* Mol Endocrinol, 1990. **4**(5): p. 721-6.
323. Attardi, B., et al., *Comparison of the effects of cycloheximide and inhibin on the gonadotropin subunit messenger ribonucleic acids.* Endocrinology, 1991. **128**(1): p. 119-25.
324. Makanji, Y., et al., *Inhibin B is a more potent suppressor of rat follicle-stimulating hormone release than inhibin a in vitro and in vivo.* Endocrinology, 2009. **150**(10): p. 4784-93.
325. Mathews, L.S. and W.W. Vale, *Expression cloning of an activin receptor, a predicted transmembrane serine kinase.* Cell, 1991. **65**(6): p. 973-82.
326. Mathews, L.S., W.W. Vale, and C.R. Kintner, *Cloning of a second type of activin receptor and functional characterization in Xenopus embryos.* Science, 1992. **255**(5052): p. 1702-5.
327. ten Dijke, P., et al., *Characterization of type I receptors for transforming growth factor-beta and activin.* Science, 1994. **264**(5155): p. 101-4.
328. Xu, J., et al., *Inhibin antagonizes inhibition of liver cell growth by activin by a dominant-negative mechanism.* J Biol Chem, 1995. **270**(11): p. 6308-13.
329. Martens, J.W., et al., *Inhibin interferes with activin signaling at the level of the activin receptor complex in Chinese hamster ovary cells.* Endocrinology, 1997. **138**(7): p. 2928-36.
330. Gray, P.C., L.M. Bilezikjian, and W. Vale, *Antagonism of activin by inhibin and inhibin receptors: a functional role for betaglycan.* Mol Cell Endocrinol, 2002. **188**(1-2): p. 254-60.
331. Lewis, K.A., et al., *Betaglycan binds inhibin and can mediate functional antagonism of activin signalling.* Nature, 2000. **404**(6776): p. 411-4.
332. Esch, F.S., et al., *Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad.* Mol Endocrinol, 1987. **1**(11): p. 849-55.

333. Ying, S.Y., et al., *Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro.* Biochem Biophys Res Commun, 1987. **149**(1): p. 133-9.
334. Thompson, T.B., et al., *The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding.* Dev Cell, 2005. **9**(4): p. 535-43.
335. Cash, J.N., et al., *The structure of myostatin:follistatin 288: insights into receptor utilization and heparin binding.* EMBO J, 2009. **28**(17): p. 2662-76.
336. Hashimoto, O., et al., *A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. Endocytotic degradation of activin and its acceleration by follistatin associated with cell-surface heparan sulfate.* J Biol Chem, 1997. **272**(21): p. 13835-42.
337. Besecke, L.M., et al., *Pituitary follistatin regulates activin-mediated production of follicle-stimulating hormone during the rat estrous cycle.* Endocrinology, 1997. **138**(7): p. 2841-8.
338. Kogawa, K., et al., *Activin-binding protein is present in pituitary.* Endocrinology, 1991. **128**(3): p. 1434-40.
339. Kaiser, U.B., et al., *Follistatin gene expression in the pituitary: localization in gonadotropes and folliculostellate cells in diestrous rats.* Endocrinology, 1992. **130**(5): p. 3048-56.
340. Bilezikjian, L.M., et al., *Activin-A regulates follistatin secretion from cultured rat anterior pituitary cells.* Endocrinology, 1993. **133**(6): p. 2554-60.
341. Lee, B.L., G. Unabia, and G. Childs, *Expression of follistatin mRNA by somatotropes and mammotropes early in the rat estrous cycle.* J Histochem Cytochem, 1993. **41**(7): p. 955-60.
342. Gospodarowicz, D. and K. Lau, *Pituitary follicular cells secrete both vascular endothelial growth factor and follistatin.* Biochem Biophys Res Commun, 1989. **165**(1): p. 292-8.
343. Bilezikjian, L.M., et al., *Rat anterior pituitary folliculostellate cells are targets of interleukin-1beta and a major source of intrapituitary follistatin.* Endocrinology, 2003. **144**(2): p. 732-40.
344. Michel, U., A. Albiston, and J.K. Findlay, *Rat follistatin: gonadal and extragonadal expression and evidence for alternative splicing.* Biochem Biophys Res Commun, 1990. **173**(1): p. 401-7.
345. Schneyer, A.L., et al., *Differential distribution of follistatin isoforms: application of a new FS315-specific immunoassay.* J Clin Endocrinol Metab, 2004. **89**(10): p. 5067-75.
346. Keutmann, H.T., A.L. Schneyer, and Y. Sidis, *The role of follistatin domains in follistatin biological action.* Mol Endocrinol, 2004. **18**(1): p. 228-40.
347. Denissova, N.G., et al., *Transforming growth factor beta -inducible independent binding of SMAD to the Smad7 promoter.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6397-402.
348. Bilezikjian, L.M., et al., *Regulation and actions of Smad7 in the modulation of activin, inhibin, and transforming growth factor-beta signaling in anterior pituitary cells.* Endocrinology, 2001. **142**(3): p. 1065-72.
349. Inman, G.J., et al., *SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7.* Mol Pharmacol, 2002. **62**(1): p. 65-74.
350. Urist, M.R., *Bone: formation by autoinduction.* Science, 1965. **150**(698): p. 893-9.
351. McDonald, N.Q. and W.A. Hendrickson, *A structural superfamily of growth factors containing a cystine knot motif.* Cell, 1993. **73**(3): p. 421-4.
352. Murray-Rust, J., et al., *Topological similarities in TGF-beta 2, PDGF-BB and NGF define a superfamily of polypeptide growth factors.* Structure, 1993. **1**(2): p. 153-9.
353. Scheufler, C., W. Sebald, and M. Hulsmeyer, *Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution.* J Mol Biol, 1999. **287**(1): p. 103-15.
354. Israel, D.I., et al., *Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo.* Growth Factors, 1996. **13**(3-4): p. 291-300.

355. Little, S.C. and M.C. Mullins, *Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis.* Nat Cell Biol, 2009. **11**(5): p. 637-43.
356. Allendorph, G.P., et al., *BMP-3 and BMP-6 structures illuminate the nature of binding specificity with receptors.* Biochemistry, 2007. **46**(43): p. 12238-47.
357. Brown, M.A., et al., *Crystal structure of BMP-9 and functional interactions with pro-region and receptors.* J Biol Chem, 2005. **280**(26): p. 25111-8.
358. Griffith, D.L., et al., *Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor beta superfamily.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 878-83.
359. Schreuder, H., et al., *Crystal structure of recombinant human growth and differentiation factor 5: evidence for interaction of the type I and type II receptor-binding sites.* Biochem Biophys Res Commun, 2005. **329**(3): p. 1076-86.
360. Kirsch, T., J. Nickel, and W. Sebald, *BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II.* EMBO J, 2000. **19**(13): p. 3314-24.
361. Kirsch, T., W. Sebald, and M.K. Dreyer, *Crystal structure of the BMP-2-BRIA ectodomain complex.* Nat Struct Biol, 2000. **7**(6): p. 492-6.
362. Weber, D., et al., *A silent H-bond can be mutationally activated for high-affinity interaction of BMP-2 and activin type IIB receptor.* BMC Struct Biol, 2007. **7**: p. 6.
363. ten Dijke, P., et al., *Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4.* J Biol Chem, 1994. **269**(25): p. 16985-8.
364. Ebisawa, T., et al., *Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation.* J Cell Sci, 1999. **112 (Pt 20)**: p. 3519-27.
365. Nishitoh, H., et al., *Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5.* J Biol Chem, 1996. **271**(35): p. 21345-52.
366. David, L., et al., *Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells.* Blood, 2007. **109**(5): p. 1953-61.
367. Scharpfenecker, M., et al., *BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis.* J Cell Sci, 2007. **120**(Pt 6): p. 964-72.
368. Yu, P.B., et al., *Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells.* J Biol Chem, 2005. **280**(26): p. 24443-50.
369. Rosenzweig, B.L., et al., *Cloning and characterization of a human type II receptor for bone morphogenetic proteins.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7632-6.
370. Gilboa, L., et al., *Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors.* Mol Biol Cell, 2000. **11**(3): p. 1023-35.
371. Nohe, A., et al., *The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways.* J Biol Chem, 2002. **277**(7): p. 5330-8.
372. Arnold, S.J., et al., *Dose-dependent Smad1, Smad5 and Smad8 signaling in the early mouse embryo.* Dev Biol, 2006. **296**(1): p. 104-18.
373. Pangas, S.A., et al., *Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice.* Mol Cell Biol, 2008. **28**(1): p. 248-57.
374. Miyazono, K., *Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins.* Bone, 1999. **25**(1): p. 91-3.
375. Miyazawa, K., et al., *Two major Smad pathways in TGF-beta superfamily signalling.* Genes Cells, 2002. **7**(12): p. 1191-204.
376. BabuRajendran, N., et al., *Structure of Smad1 MH1/DNA complex reveals distinctive rearrangements of BMP and TGF-beta effectors.* Nucleic Acids Res, 2010. **38**(10): p. 3477-88.

377. Lopez-Rovira, T., et al., *Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene.* J Biol Chem, 2002. **277**(5): p. 3176-85.
378. Karaulanov, E., W. Knochel, and C. Niehrs, *Transcriptional regulation of BMP4 synexpression in transgenic Xenopus.* EMBO J, 2004. **23**(4): p. 844-56.
379. Shepherd, T.G., B.L. Theriault, and M.W. Nachtigal, *Autocrine BMP4 signalling regulates ID3 proto-oncogene expression in human ovarian cancer cells.* Gene, 2008. **414**(1-2): p. 95-105.
380. Takase, M., et al., *Induction of Smad6 mRNA by bone morphogenetic proteins.* Biochem Biophys Res Commun, 1998. **244**(1): p. 26-9.
381. Miyazono, K. and K. Miyazawa, *Id: a target of BMP signaling.* Sci STKE, 2002. **2002**(151): p. pe40.
382. Abe, J., *Bone morphogenetic protein (BMP) family, SMAD signaling and Id helix-loop-helix proteins in the vasculature: the continuous mystery of BMPs pleotropic effects.* J Mol Cell Cardiol, 2006. **41**(1): p. 4-7.
383. Coppe, J.P., A.P. Smith, and P.Y. Desprez, *Id proteins in epithelial cells.* Exp Cell Res, 2003. **285**(1): p. 131-45.
384. Katagiri, T., et al., *Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage.* J Cell Biol, 1994. **127**(6 Pt 1): p. 1755-66.
385. Kowanetz, M., et al., *Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein.* Mol Cell Biol, 2004. **24**(10): p. 4241-54.
386. Locklin, R.M., et al., *Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology.* J Bone Miner Res, 2001. **16**(12): p. 2192-204.
387. Peng, Y., et al., *Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells.* J Biol Chem, 2004. **279**(31): p. 32941-9.
388. Samanta, J. and J.A. Kessler, *Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation.* Development, 2004. **131**(17): p. 4131-42.
389. Rosenfeld, M.G., et al., *Multistep signaling and transcriptional requirements for pituitary organogenesis in vivo.* Recent Prog Horm Res, 2000. **55**: p. 1-13; discussion 13-4.
390. Takuma, N., et al., *Formation of Rathke's pouch requires dual induction from the diencephalon.* Development, 1998. **125**(23): p. 4835-40.
391. Ericson, J., et al., *Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary.* Development, 1998. **125**(6): p. 1005-15.
392. Xia, Y., et al., *Localization and action of Dragon (repulsive guidance molecule b), a novel bone morphogenetic protein coreceptor, throughout the reproductive axis.* Endocrinology, 2005. **146**(8): p. 3614-21.
393. Carroll, R.S., et al., *Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels.* Mol Endocrinol, 1989. **3**(12): p. 1969-76.
394. DePaolo, L.V., et al., *In vivo comparison of the follicle-stimulating hormone-suppressing activity of follistatin and inhibin in ovariectomized rats.* Endocrinology, 1991. **128**(2): p. 668-74.
395. Wiater, E. and W. Vale, *Inhibin is an antagonist of bone morphogenetic protein signaling.* J Biol Chem, 2003. **278**(10): p. 7934-41.
396. Wiater, E., et al., *Identification of distinct inhibin and transforming growth factor beta-binding sites on betaglycan: functional separation of betaglycan co-receptor actions.* J Biol Chem, 2006. **281**(25): p. 17011-22.

397. Yamashita, H., et al., *Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects.* J Cell Biol, 1995. **130**(1): p. 217-26.
398. Iemura, S., et al., *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 U S A, 1998. **95**(16): p. 9337-42.
399. Lopez-Casillas, F., et al., *Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system.* Cell, 1991. **67**(4): p. 785-95.
400. Huang, H.J., et al., *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, 2001. **142**(6): p. 2267-74.
401. Huang, H.J., et al., *A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone.* Endocrinology, 2001. **142**(6): p. 2275-83.
402. Gore, A.J., et al., *Differential regulation of follicle stimulating hormone by activin A and TGFB1 in murine gonadotropes.* Reprod Biol Endocrinol, 2005. **3**: p. 73.
403. Dale, L., et al., *Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development.* Development, 1992. **115**(2): p. 573-85.
404. Jones, C.M., et al., *DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in Xenopus mesoderm induction.* Development, 1992. **115**(2): p. 639-47.
405. Otsuka, F. and S. Shimasaki, *A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes.* Endocrinology, 2002. **143**(12): p. 4938-41.
406. Galloway, S.M., et al., *Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner.* Nat Genet, 2000. **25**(3): p. 279-83.
407. Takeda, M., et al., *Involvement of activin/BMP system in development of human pituitary gonadotropinomas and nonfunctioning adenomas.* Biochem Biophys Res Commun, 2003. **306**(4): p. 812-8.
408. Faure, M.O., et al., *BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary.* J Endocrinol, 2005. **186**(1): p. 109-21.
409. Nicol, L., et al., *Bone morphogenetic protein-4 interacts with activin and GnRH to modulate gonadotrophin secretion in LbetaT2 gonadotrophs.* J Endocrinol, 2008. **196**(3): p. 497-507.
410. Sallon, C., et al., *Dynamic regulation of pituitary mRNAs for BMP-4, BMP receptors and activin/inhibin subunits in the ewe during the oestrous cycle and in cultured pituitary cells.* J Endocrinol, 2010.
411. McNatty, K.P., et al., *Genetic mutations influencing ovulation rate in sheep.* Reprod Fertil Dev, 2001. **13**(7-8): p. 549-55.
412. Monget, P., et al., *Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals.* Domest Anim Endocrinol, 2002. **23**(1-2): p. 139-54.
413. Mulsant, P., et al., *Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5104-9.
414. Fabre, S., et al., *The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality.* J Endocrinol, 2003. **177**(3): p. 435-44.
415. Souza, C.J., et al., *The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene.* J Endocrinol, 2001. **169**(2): p. R1-6.
416. Wilson, T., et al., *Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells.* Biol Reprod, 2001. **64**(4): p. 1225-35.
417. Campbell, B.K., et al., *Enhanced response of granulosa and theca cells from sheep carriers of the FecB mutation in vitro to gonadotropins and bone morphogenic protein-2, -4, and -6.* Endocrinology, 2006. **147**(4): p. 1608-20.

418. Young, J.M., et al., *The activin receptor-like kinase 6 Booroola mutation enhances suppressive effects of bone morphogenetic protein 2 (BMP2), BMP4, BMP6 and growth and differentiation factor-9 on FSH release from ovine primary pituitary cell cultures.* J Endocrinol, 2008. **196**(2): p. 251-61.
419. Shim, J.H., et al., *TAK1 is an essential regulator of BMP signalling in cartilage.* EMBO J, 2009. **28**(14): p. 2028-41.
420. Yamaguchi, K., et al., *XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway.* EMBO J, 1999. **18**(1): p. 179-87.
421. Shibuya, H., et al., *Role of TAK1 and TAB1 in BMP signaling in early Xenopus development.* EMBO J, 1998. **17**(4): p. 1019-28.
422. Gazzerro, E. and E. Canalis, *Bone morphogenetic proteins and their antagonists.* Rev Endocr Metab Disord, 2006. **7**(1-2): p. 51-65.
423. Kameda, T., et al., *Developmental patterning in chondrocytic cultures by morphogenic gradients: BMP induces expression of indian hedgehog and noggin.* Genes Cells, 1999. **4**(3): p. 175-84.
424. Groppe, J., et al., *Structural basis of BMP signaling inhibition by Noggin, a novel twelve-membered cystine knot protein.* J Bone Joint Surg Am, 2003. **85-A Suppl 3**: p. 52-8.
425. Zhou, G., et al., *Role of AMP-activated protein kinase in mechanism of metformin action.* J Clin Invest, 2001. **108**(8): p. 1167-74.
426. Yu, P.B., et al., *Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism.* Nat Chem Biol, 2008. **4**(1): p. 33-41.
427. Wrighton, K.H., et al., *Transforming Growth Factor {beta} Can Stimulate Smad1 Phosphorylation Independently of Bone Morphogenic Protein Receptors.* J Biol Chem, 2009. **284**(15): p. 9755-63.
428. Boergermann, J.H., et al., *Dorsomorphin and LDN-193189 inhibit BMP-mediated Smad, p38 and Akt signalling in C2C12 cells.* Int J Biochem Cell Biol, 2010.
429. Cuny, G.D., et al., *Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors.* Bioorg Med Chem Lett, 2008. **18**(15): p. 4388-92.
430. *CG11 Fertility: information for the public*. Available from: http://www.nice.org.uk/guidance/index.jsp?action=download&o=29271.
431. *Regulated fertility services: a commissioning aid - June 2009*. Available from: http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_101070.
432. *Unexplained Infertility Background, Tests and Treatment Options*. Available from: http://www.advancedfertility.com/unexplai.htm.
433. McNatty, K.P., et al., *Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate.* J Reprod Fertil, 1987. **80**(2): p. 577-88.
434. Harrison, C.A., et al., *Antagonists of activin signaling: mechanisms and potential biological applications.* Trends Endocrinol Metab, 2005. **16**(2): p. 73-8.
435. Allendorph, G.P., W.W. Vale, and S. Choe, *Structure of the ternary signaling complex of a TGF-beta superfamily member.* Proc Natl Acad Sci U S A, 2006. **103**(20): p. 7643-8.
436. Shimasaki, S., et al., *The bone morphogenetic protein system in mammalian reproduction.* Endocr Rev, 2004. **25**(1): p. 72-101.
437. Davis GH, M.G., Allision AJ, Kelly RW & Bray AR *Segregation of a major gene influencing fecundity in progeny of Booroola sheep.* New Zealand Journal of Agricultural Research, 1982 **25**: p. 525–529.
438. Piper LR, B.B.D.G., *The single gene inheritance of the high litter size of the Booroola Merino.* In Genetics of Reproduction in Sheep, 1985 p. 115–125.

439. Campbell, B.K., et al., *The FecB (Booroola) gene acts at the ovary: in vivo evidence.* Reproduction, 2003. **126**(1): p. 101-11.
440. Clark, S.J., et al., *High sensitivity mapping of methylated cytosines.* Nucleic Acids Res, 1994. **22**(15): p. 2990-7.
441. Frommer, M., et al., *A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands.* Proc Natl Acad Sci U S A, 1992. **89**(5): p. 1827-31.
442. Liu, F., et al., *Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs.* Mol Cell Biol, 1995. **15**(7): p. 3479-86.
443. Hata, A., et al., *OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways.* Cell, 2000. **100**(2): p. 229-40.
444. Itoh, S., et al., *Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses.* J Biol Chem, 2003. **278**(6): p. 3751-61.
445. Clouaire, T. and I. Stancheva, *Methyl-CpG binding proteins: specialized transcriptional repressors or structural components of chromatin?* Cell Mol Life Sci, 2008. **65**(10): p. 1509-22.
446. Kawabata, M., A. Chytil, and H.L. Moses, *Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-beta receptor.* J Biol Chem, 1995. **270**(10): p. 5625-30.
447. Bindon, B.M., *Reproductive biology of the Booroola Merino sheep.* Aust J Biol Sci, 1984. **37**(3): p. 163-89.
448. Robertson, D.M., et al., *Pituitary gonadotrophins in Booroola and control Merino sheep.* J Reprod Fertil, 1984. **71**(1): p. 189-97.
449. Conway, G.S., *Clinical manifestations of genetic defects affecting gonadotrophins and their receptors.* Clin Endocrinol (Oxf), 1996. **45**(6): p. 657-63.
450. Chan, W.Y., *Molecular genetic, biochemical, and clinical implications of gonadotropin receptor mutations.* Mol Genet Metab, 1998. **63**(2): p. 75-84.
451. Kumar, T.R., *What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice?* Reproduction, 2005. **130**(3): p. 293-302.
452. Lei, Z.M., et al., *Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene.* Mol Endocrinol, 2001. **15**(1): p. 184-200.
453. Winters, S.J. and J.P. Moore, *Intra-pituitary regulation of gonadotrophs in male rodents and primates.* Reproduction, 2004. **128**(1): p. 13-23.
454. Ho, C.C. and D.J. Bernard, *Bone morphogenetic protein 2 signals via BMPR1A to regulate murine follicle-stimulating hormone beta subunit transcription.* Biol Reprod, 2009. **81**(1): p. 133-41.
455. Filyak, Y., et al., *Doxorubicin inhibits TGF-beta signaling in human lung carcinoma A549 cells.* Eur J Pharmacol, 2008. **590**(1-3): p. 67-73.
456. Dennler, S., et al., *Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene.* EMBO J, 1998. **17**(11): p. 3091-100.
457. Lee, J., et al., *Id helix-loop-helix proteins negatively regulate TRANCE-mediated osteoclast differentiation.* Blood, 2006. **107**(7): p. 2686-93.
458. VanGuilder, H.D., K.E. Vrana, and W.M. Freeman, *Twenty-five years of quantitative PCR for gene expression analysis.* Biotechniques, 2008. **44**(5): p. 619-26.
459. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
460. Zhang, Y., T. Musci, and R. Derynck, *The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function.* Curr Biol, 1997. **7**(4): p. 270-6.
461. Lyden, D., et al., *Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts.* Nature, 1999. **401**(6754): p. 670-7.

462. Yan, W., et al., *High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice.* Mol Cell Biol, 1997. **17**(12): p. 7317-27.
463. Kim, J.K., M. Takeuchi, and Y. Yokota, *Impairment of intestinal intraepithelial lymphocytes in Id2 deficient mice.* Gut, 2004. **53**(4): p. 480-6.
464. Rivera, R.R., et al., *Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3.* Immunity, 2000. **12**(1): p. 17-26.
465. Liu, D., B.L. Black, and R. Derynck, *TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3.* Genes Dev, 2001. **15**(22): p. 2950-66.
466. Wong, C., et al., *Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta.* Mol Cell Biol, 1999. **19**(3): p. 1821-30.
467. Chen, X., M.J. Rubock, and M. Whitman, *A transcriptional partner for MAD proteins in TGF-beta signalling.* Nature, 1996. **383**(6602): p. 691-6.
468. Chen, X., et al., *Smad4 and FAST-1 in the assembly of activin-responsive factor.* Nature, 1997. **389**(6646): p. 85-9.
469. Zhang, Y., X.H. Feng, and R. Derynck, *Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription.* Nature, 1998. **394**(6696): p. 909-13.
470. Hanai, J., et al., *Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter.* J Biol Chem, 1999. **274**(44): p. 31577-82.
471. Chen, C.R., et al., *E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression.* Cell, 2002. **110**(1): p. 19-32.
472. Cordenonsi, M., et al., *Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads.* Cell, 2003. **113**(3): p. 301-14.
473. Seoane, J., et al., *Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation.* Cell, 2004. **117**(2): p. 211-23.
474. Grinberg, A.V. and T. Kerppola, *Both Max and TFE3 cooperate with Smad proteins to bind the plasminogen activator inhibitor-1 promoter, but they have opposite effects on transcriptional activity.* J Biol Chem, 2003. **278**(13): p. 11227-36.
475. Chen, X., et al., *Upregulation of ID protein by growth and differentiation factor 5 (GDF5) through a smad-dependent and MAPK-independent pathway in HUVSMC.* J Mol Cell Cardiol, 2006. **41**(1): p. 26-33.
476. Kaneko, K., et al., *Endothelial expression of bone morphogenetic protein receptor type 1a is required for atrioventricular valve formation.* Ann Thorac Surg, 2008. **85**(6): p. 2090-8.
477. Katagiri, T., et al., *Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis.* Genes Cells, 2002. **7**(9): p. 949-60.
478. Tian, F., et al., *Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro.* Biochem Biophys Res Commun, 2010. **392**(3): p. 283-8.
479. Valdimarsdottir, G., et al., *Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells.* Circulation, 2002. **106**(17): p. 2263-70.
480. Yang, J., et al., *Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension.* Circ Res, 2008. **102**(10): p. 1212-21.
481. Yang, J., et al., *Smad-dependent and smad-independent induction of id1 by prostacyclin analogues inhibits proliferation of pulmonary artery smooth muscle cells in vitro and in vivo.* Circ Res, 2010. **107**(2): p. 252-62.
482. Yu, P.B., et al., *Bone morphogenetic protein (BMP) type II receptor is required for BMP-mediated growth arrest and differentiation in pulmonary artery smooth muscle cells.* J Biol Chem, 2008. **283**(7): p. 3877-88.

483. Korchynskyi, O. and P. ten Dijke, *Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter.* J Biol Chem, 2002. **277**(7): p. 4883-91.
484. Vinals, F., et al., *BMP-2 decreases Mash1 stability by increasing Id1 expression.* EMBO J, 2004. **23**(17): p. 3527-37.
485. Hua, H., et al., *BMP4 regulates pancreatic progenitor cell expansion through Id2.* J Biol Chem, 2006. **281**(19): p. 13574-80.
486. Karaya, K., et al., *Regulation of Id2 expression by CCAAT/enhancer binding protein beta.* Nucleic Acids Res, 2005. **33**(6): p. 1924-34.
487. Yeh, K. and R.W. Lim, *Genomic organization and promoter analysis of the murine Id3 gene.* Gene, 2000. **254**(1-2): p. 163-71.
488. Mishina, Y., et al., *Generation of Bmpr/Alk3 conditional knockout mice.* Genesis, 2002. **32**(2): p. 69-72.
489. Therrien, M. and J. Drouin, *Cell-specific helix-loop-helix factor required for pituitary expression of the pro-opiomelanocortin gene.* Mol Cell Biol, 1993. **13**(4): p. 2342-53.
490. Ho, C.C. and D.J. Bernard, *Bone Morphogenetic Protein 2 Acts via Inhibitor of DNA Binding Proteins to Synergistically Regulate Follicle-Stimulating Hormone {beta} Transcription with Activin A.* Endocrinology, 2010.
491. Hoodless, P.A., et al., *MADR1, a MAD-related protein that functions in BMP2 signaling pathways.* Cell, 1996. **85**(4): p. 489-500.
492. Belaguli, N.S., et al., *Cooperation between GATA4 and TGF-beta signaling regulates intestinal epithelial gene expression.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(6): p. G1520-33.
493. Christy, B.A., et al., *An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene.* Proc Natl Acad Sci U S A, 1991. **88**(5): p. 1815-9.
494. Barone, M.V., et al., *Id proteins control growth induction in mammalian cells.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4985-8.
495. Liu, J., S. Wilson, and T. Reh, *BMP receptor 1b is required for axon guidance and cell survival in the developing retina.* Dev Biol, 2003. **256**(1): p. 34-48.
496. Miura, S., A.P. Singh, and Y. Mishina, *Bmpr1a is required for proper migration of the AVE through regulation of Dkk1 expression in the pre-streak mouse embryo.* Dev Biol, 2010. **341**(1): p. 246-54.
497. Davis, B.N., et al., *SMAD proteins control DROSHA-mediated microRNA maturation.* Nature, 2008. **454**(7200): p. 56-61.
498. Kim, J., et al., *Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic.* Nature, 1997. **388**(6639): p. 304-8.
499. Xu, X., et al., *Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm.* Genes Dev, 1998. **12**(15): p. 2354-70.
500. Kusanagi, K., et al., *Characterization of a bone morphogenetic protein-responsive Smad-binding element.* Mol Biol Cell, 2000. **11**(2): p. 555-65.
501. Nakahiro, T., et al., *Identification of BMP responsive elements in the mouse Id2 gene.* Biochem Biophys Res Commun, 2010.
502. Lai, C.F. and S.L. Cheng, *Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells.* J Biol Chem, 2002. **277**(18): p. 15514-22.
503. Lee-Hoeflich, S.T., et al., *Activation of LIMK1 by binding to the BMP receptor, BMPRII, regulates BMP-dependent dendritogenesis.* EMBO J, 2004. **23**(24): p. 4792-801.
504. Chan, M.C., et al., *A novel regulatory mechanism of the bone morphogenetic protein (BMP) signaling pathway involving the carboxyl-terminal tail domain of BMP type II receptor.* Mol Cell Biol, 2007. **27**(16): p. 5776-89.

505. Sun, X.H., et al., *Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins.* Mol Cell Biol, 1991. **11**(11): p. 5603-11.
506. Ying, Q.L., et al., *BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3.* Cell, 2003. **115**(3): p. 281-92.
507. Young, K.H., *Yeast two-hybrid: so many interactions, (in) so little time.* Biol Reprod, 1998. **58**(2): p. 302-11.
508. Warner, D.R., et al., *Identification of novel Smad binding proteins.* Biochem Biophys Res Commun, 2003. **312**(4): p. 1185-90.
509. Brown, K.A., et al., *Identification of novel Smad2 and Smad3 associated proteins in response to TGF-beta1.* J Cell Biochem, 2008. **105**(2): p. 596-611.
510. Knuesel, M., et al., *Identification of novel protein-protein interactions using a versatile mammalian tandem affinity purification expression system.* Mol Cell Proteomics, 2003. **2**(11): p. 1225-33.
511. Puig, O., et al., *The tandem affinity purification (TAP) method: a general procedure of protein complex purification.* Methods, 2001. **24**(3): p. 218-29.
512. Rigaut, G., et al., *A generic protein purification method for protein complex characterization and proteome exploration.* Nat Biotechnol, 1999. **17**(10): p. 1030-2.
513. Urh, M., D. Simpson, and K. Zhao, *Affinity chromatography: general methods.* Methods Enzymol, 2009. **463**: p. 417-38.
514. Brymora, A., V.A. Valova, and P.J. Robinson, *Protein-protein interactions identified by pull-down experiments and mass spectrometry.* Curr Protoc Cell Biol, 2004. **Chapter 17**: p. Unit 17 5.
515. Hellman, L.M. and M.G. Fried, *Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions.* Nat Protoc, 2007. **2**(8): p. 1849-61.
516. Joung, J.K., E.I. Ramm, and C.O. Pabo, *A bacterial two-hybrid selection system for studying protein-DNA and protein-protein interactions.* Proc Natl Acad Sci U S A, 2000. **97**(13): p. 7382-7.
517. Mantani, A., et al., *The mouse Id2 and Id4 genes: structural organization and chromosomal localization.* Gene, 1998. **222**(2): p. 229-35.
518. Haugen, M.J. and A. Johnson, *Bone morphogenetic protein 2 (BMP2) inhibits FSH responsiveness in hen granulosa cells.* Reproduction, 2010.
519. Kersten, C., et al., *BMP-6 inhibits human bone marrow B lymphopoiesis--upregulation of Id1 and Id3.* Exp Hematol, 2006. **34**(1): p. 72-81.
520. Liu, T., et al., *BMP-2 promotes differentiation of osteoblasts and chondroblasts in Runx2-deficient cell lines.* J Cell Physiol, 2007. **211**(3): p. 728-35.
521. Pagliuca, A., P. Cannada-Bartoli, and L. Lania, *A role for Sp and helix-loop-helix transcription factors in the regulation of the human Id4 gene promoter activity.* J Biol Chem, 1998. **273**(13): p. 7668-74.
522. Cheng, X., et al., *Bone morphogenetic protein signaling and olig1/2 interact to regulate the differentiation and maturation of adult oligodendrocyte precursor cells.* Stem Cells, 2007. **25**(12): p. 3204-14.
523. Miyoshi, T., et al., *Involvement of bone morphogenetic protein-4 in GH regulation by octreotide and bromocriptine in rat pituitary GH3 cells.* J Endocrinol, 2008. **197**(1): p. 159-69.
524. Tsukamoto, N., et al., *Effects of bone morphogenetic protein (BMP) on adrenocorticotropin production by pituitary corticotrope cells: involvement of up-regulation of BMP receptor signaling by somatostatin analogs.* Endocrinology, 2010. **151**(3): p. 1129-41.
525. Sauer, B. and N. Henderson, *Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1.* Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5166-70.
526. Muller, U., *Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis.* Mech Dev, 1999. **82**(1-2): p. 3-21.

527. Wen, S., et al., *Functional characterization of genetically labeled gonadotropes.* Endocrinology, 2008. **149**(6): p. 2701-11.

Appendix

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Bone Morphogenetic Protein 2 Signals via BMPR1A to Regulate Murine Follicle-Stimulating Hormone Beta Subunit Transcription[1]

Catherine C. Ho and Daniel J. Bernard[2]

Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

ABSTRACT

Follicle-stimulating hormone beta subunit (*Fshb*) expression is regulated by transforming growth factor beta superfamily ligands. Recently, we demonstrated that bone morphogenetic proteins (BMPs) stimulate *Fshb* transcription alone and in synergy with activins. Also, transfection of the BMP type II receptor (BMPR2) and constitutively active forms of the type I receptors (activin A receptor type I [ACVR1] or BMP receptor type IA [BMPR1A]) in immortalized gonadotroph cells (LbetaT2) stimulated murine *Fshb* promoter-reporter activity. A third type I receptor (BMP receptor type IB [BMPR1B]) is also expressed in LbetaT2 cells, but we did not previously assess its functional role. A point mutation in BMPR1B (Q249R) is associated with increased ovulation rates and elevated FSH levels in Booroola (*FecB*) sheep. Herein, we assessed whether BMPR1B can regulate *Fshb* transcription in LbetaT2 cells and whether its ability to do so is altered by the Q249R mutation. As with ACVR1 and BMPR1A, coexpression of BMPR1B with BMPR2 increased *Fshb* promoter-reporter activity in BMP2-dependent and BMP2-independent fashions. Unexpectedly, the BMPR1B-Q249R mutant was equivalent to the wild type in its ability to stimulate SMAD1/5 phosphorylation and *Fshb* transcription. Pharmacological inhibition of ACVR1, BMPR1A, and BMPR1B confirmed that one or more of these receptors are required for BMP2-stimulated SMAD1/5 phosphorylation and *Fshb* reporter activity. Knockdown of endogenous BMPR1A, but not ACVR1 or BMPR1B, significantly impaired the synergism of BMP2 with activin A. Collectively, these data suggest that BMPR1A is the preferred BMP2 type I receptor in LbetaT2 cells and that neither ACVR1 nor BMPR1B compensates for its loss. The specific mechanism(s) through which the Booroola *FecB* mutation alters BMPR1B function remains to be determined.

ALK3, ALK6, anterior pituitary, BMP, BMPR2, Booroola, FecB, *follicle-stimulating hormone, FSH, gonadotroph, pituitary, signal transduction, SMAD, TGFB*

INTRODUCTION

The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are essential reproductive hormones. Both are secreted from gonadotrophs of the anterior pituitary, but the two act to regulate different aspects of gonadal function. The gonadotropins are heterodimeric glycoproteins (α/β), with their β subunits determining rates of mature hormone synthesis and biological specificity. Both FSH and LH are regulated by gonadotropin-releasing hormone secreted from the hypothalamus and gonadal sex steroids; however, endocrine-paracrine transforming growth factor beta (TGFB) superfamily ligands such as activins and inhibins act to selectively regulate FSH synthesis. Activins signal via a combination of type II receptors (ACVR2 or ACVR2B), type I receptors (ACVR1B and ACVR1C, also known as activin receptor-like kinase [ALK] 4 and ALK7), and downstream signaling effectors (SMAD2 and SMAD3) to up-regulate FSH beta subunit (*Fshb*) transcription [1–5]. In contrast, inhibins suppress *Fshb* expression by blocking the actions of activins through a competitive binding mechanism [6].

Recently, other members of the TGFB superfamily, the bone morphogenetic proteins (BMPs), were shown to stimulate *Fshb* transcription alone and in synergy with activins [7–10]. The BMPs are expressed in LβT2 cells (an immortalized murine gonadotroph cell line) and in adult murine pituitary [7–9] and might regulate FSH synthesis in vivo. We previously reported [7] that BMP6 and BMP7, although endogenously expressed in LβT2 cells, only modestly regulate *Fshb* transcription. In contrast, BMP2 and BMP4 stimulate *Fshb* transcription more potently, but their expression in LβT2 cells is very low. However, BMP2 and BMP4 are highly expressed in the murine pituitary and may act as paracrine regulators of gonadotroph function. Relative to equimolar activins, BMP2 and BMP4 only weakly stimulate *Fshb* transcription, but they are nonetheless potent synergistic regulators when applied in combination with the activins. Physiologically, BMPs may be more important in terms of their cooperative rather than independent actions.

BMP2 and BMP4 signaling is initiated by the interaction of the ligands with BMP type I receptors such as BMPR1A and BMPR1B (also known as ALK3 and ALK6). A type II receptor such as BMP type II receptor (BMPR2) is then recruited into the complex and phosphorylates the type I receptors [11, 12]. The activated type I receptors then phosphorylate intracellular signaling proteins, the most thoroughly characterized of which are the receptor-regulated SMADs (R-SMADs) SMAD1, SMAD5, and SMAD8. Once phosphorylated, R-SMADs form heteromeric complexes with the coregulatory SMAD (SMAD4), accumulate in the nucleus, and act as transcription factors, either activating or repressing gene expression [11, 13, 14]. Activins stimulate FSH synthesis by up-regulating *Fshb* subunit gene transcription at least in part through the SMAD2 and SMAD3 signaling proteins [2–4, 15]. The available data suggest that BMP2 might preferentially signal through SMAD8 to regulate the *Fshb* gene [7].

The BMP family members show some promiscuity in their binding to type I and type II receptors within the TGFB superfamily. For example, BMP2 and BMP4 preferentially signal through the type II receptor BMPR2 but can use ACVR2A in its absence [16]. Similarly, BMPs can bind to

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[2]Daniel J. Bernard, Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada H3G 1Y6.
FAX: 514 398 6705; e-mail: daniel.bernard@mcgill.ca

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several type I receptors, including ACVR1, BMPR1A, and BMPR1B [17]. Each of these type I receptors is expressed in LβT2 cells [7, 9, 18]; however, our previous overexpression data suggested a preferred role for ACVR1 in mediating BMP2 responses [7]. Nonetheless, a role for BMPR1B was not assessed, and the data with wild-type and constitutively active BMPR1A yielded conflicting results.

A potential role for BMPR1B in FSH regulation is particularly intriguing in light of the phenotype of so-called Booroola (*FecB*) sheep. These animals show increased ovulation rates, leading to multiple births [19–21], and FSH levels are elevated in some Booroola flocks [22]. The *FecB* mutation was mapped to the *Bmpr1b* locus and a missense point mutation (CAG→CGG [Q249R]) discovered in the highly conserved intracellular serine-threonine kinase domain of the receptor [23–25]; however, the specific alteration in receptor function, at a mechanistic level, has not been determined. Some data suggest that the mutation leads to a partial loss of receptor function, particularly at the ovarian level [26, 27], but alterations at the pituitary level have not been ruled out definitively. In fact, recent data show differences in BMP signaling in pituitary cultures from Booroola and wild-type sheep [28]. These effects may not be mediated directly at the gonadotroph level, as previous investigators failed to detect BMPR1B expression in ovine gonadotrophs by immunofluorescence [29]. Nonetheless, one cannot rule out the possibility of low-level expression in these cells that evaded detection by this method. Indeed, *Bmpr1b* mRNA is expressed at low levels in LβT2 cells [7, 18]. Herein, we assessed the relative roles of endogenous ACVR1, BMPR1A, and BMPR1B in BMP2-regulated *Fshb* transcription in LβT2 cells and examined potential functional changes in the mutant BMPR1B receptor (Q249R) at the level of the gonadotroph.

MATERIALS AND METHODS

Reagents

Human recombinant activin A and BMP2 were purchased from R&D Systems (Minneapolis, MN). Wisent (St-Bruno, QC) was the supplier of gentamycin, 1× PBS, and Dulbecco modified Eagle medium (DMEM) with 4.5 g/l glucose, L-glutamine, and sodium pyruvate. We obtained 1× passive lysis buffer (PLB) from Promega (Madison, WI). Protease inhibitor cocktail tablets (CompleteMini) were purchased from Roche (Nutley, NJ). Aprotinin, leupeptin, pepstatin, PMSF, SB431542, mouse monoclonal β-actin (No. A5441), mouse monoclonal HA (No. H9658) and MYC (No. 9E10) antibodies, and rabbit monoclonal FLAG (No. F3165) antibody were from Sigma (St. Louis, MO). The pSMAD1/5/8 rabbit polyclonal antibody (No. 9511) was from Cell Signaling Technology, Inc. (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad (Hercules, CA), and enhanced chemiluminescence Plus reagent was from GE Healthcare (Piscataway, NJ). Compound C (No. 171261) was purchased from Calbiochem (San Diego, CA). The following short-interfering (si) RNAs were purchased from Dharmacon, Inc. (Lafayette, CO): control (catalog No. D-001210–05), ACVR1 (catalog No. D-042047–01), BMPR1A (catalog No. D-040598–01), BMPR1B (catalog No. D-051071–01), ACVR2 (catalog No. D-040676–01), ACVR2B (catalog No. D-040629–02), and BMPR2 (catalog No. D-040599–01). Sodium bisulfite was purchased from Fisher Scientific (Fair Lawn, NJ) (catalog No. S654–500), and quinol hydroquinone was purchased from BDH AnalaR (Poole, England) (catalog No. 10312).

Constructs

The expression constructs for rat ACVR1-HA, FLAG-ACVR2, and FLAG-ACVR2B and for human FLAG-SMAD1 were provided by Dr. Teresa Woodruff (Northwestern University, Evanston, IL). Human BMPR1A-HA (Q233D) and murine BMPR1B-HA (Q203D) were provided by Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan). The following variants were constructed by site-directed mutagenesis using the QuikChange protocol (Stratagene, La Jolla, CA) and the primers listed in Supplemental Table S1 (available at www.biolreprod.org): constitutively active and siRNA-sensitive ACVR1-HA (Q207D); wild-type and siRNA-sensitive BMPR1A-Q233D-HA; wild-type BMPR1B-HA, BMPR1B-Q249R-HA, BMPR1B-Q249R/Q203D-HA, and BMPR1B-Q203D/D265A-MYC; methylated BMPR1B-Q249R-HA; and siRNA-resistant BMPR1B-Q203D-HA. In the case of methylated BMPR1B-Q249R, primers containing methylated cytosines (Supplemental Table S1) were used, and the resulting PCR products were purified by ethanol precipitation following *Dpn*I digestion of the parental plasmid and utilized directly in transfection experiments. Methylation was confirmed by bisulfite sequencing [30, 31]. All BMPR1A and BMPR1B constructs were subcloned into pcDNA4 (Invitrogen, San Diego, CA). This removed the HA tag and replaced it with a C-terminal MYC-HIS tag. Human FLAG-SMAD5 was provided by Dr. Tetsuro Watabe (Tokyo University, Tokyo, Japan). The human BMPR2 expression construct [32] and BREX4-luc [33] were provided by Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY). The murine *Fshb* promoter-reporter constructs were described previously [4].

Cell Cultures and Transfections

Immortalized murine gonadotroph LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego, CA) and were cultured in 10% fetal bovine serum (FBS)/DMEM and 4 μg/ml gentamycin as described previously [2]. For luciferase assays, cells were plated in 24-well plates (2.5×10^5 cells/well) approximately 36 h before transfection. Cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Twenty-four hours after transfection, cells were washed in 1× PBS and then treated with 1 nM (25 ng/ml) activin A and/or BMP2 in DMEM or with DMEM alone (control) for the indicated times. In overexpression experiments, 450 ng of the reporter and 100 ng of each receptor and/or effector were used per well. Cells were placed into serum-free media 24 h after transfection. In some experiments, 10 μM SB431542, an ACVR1B/ACVR1C/TGFBRI inhibitor [34], was included to block the effects of endogenous activin B (or other ligands signaling through these receptors). In RNA interference (RNAi) experiments, siRNAs were transfected at 5 nM. Resulting data were calibrated to cells transfected with the 1× siRNA buffer only (20 mM KCl, 6 mM HEPES [pH 7.5], and 0.2 mM $MgCl_2$) or to cells transfected with the control siRNA. Lysates were collected 24 h after transfer to serum-free medium. CHO cells were obtained from Dr. Patricia Morris (Population Council, New York, NY) and were cultured in F-12/DMEM containing 10% FBS and 4 μg/ml gentamycin. Except for the BMPR1B/D265A experiment (where 4 μg FLAG-SMAD1 and 4 μg of receptor were transfected in CHO cells seeded in 10-cm plates), CHO cells in 6-well plates were transfected when 70%–80% confluent using Lipofectamine reagent, 300 ng of the indicated receptor expression vectors, and 1 μg FLAG-SMAD1 or FLAG-SMAD5 for 6 h and were then placed in growth media. The repeat of this experiment in LβT2 cells in 6-well plates was performed in a similar fashion, except that Plus reagent and 1200 ng of the indicated receptor expression vectors were included. Cell lysates were then harvested the following day. HepG2 cells (No. HB-8065) were purchased from ATCC (Manassas, VA) and were cultured in 10% FBS/Eagle minimum essential medium (modified by ATCC) and 4 μg/ml gentamycin. Transfection protocols were identical to those used for the LβT2 cells.

Luciferase Assays

Cells were washed with 1× PBS and lysed in 1× PLB. Luciferase assays were performed on an Orion II microplate luminometer (Berthold Detection Systems, Oak Ridge, TN) using standard reagents. All treatments were performed in duplicate or triplicate as described in the text or figure legends. Data presented are from at least 2–3 independent experiments.

Immunoblots

Cells were washed with 1× PBS, and whole-cell protein extracts (WCEs) were prepared with 1× RIPA (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate [pH 6.8], 2 mM edetic acid, 50 mM sodium fluoride, and CompleteMini tablets) and centrifuged at $13\,000 \times g$ for 0.5 h at 4°C to remove cellular debris. The WCEs were subjected to immunoblot analyses as previously described [2]. Briefly, equivalent amounts of protein were separated by SDS-PAGE and were transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH). Blots were probed with the indicated antibodies using standard techniques.

Statistical Analysis

Data from three replicate experiments were highly similar, and their means were pooled for statistical analyses. Data are presented as fold change from the

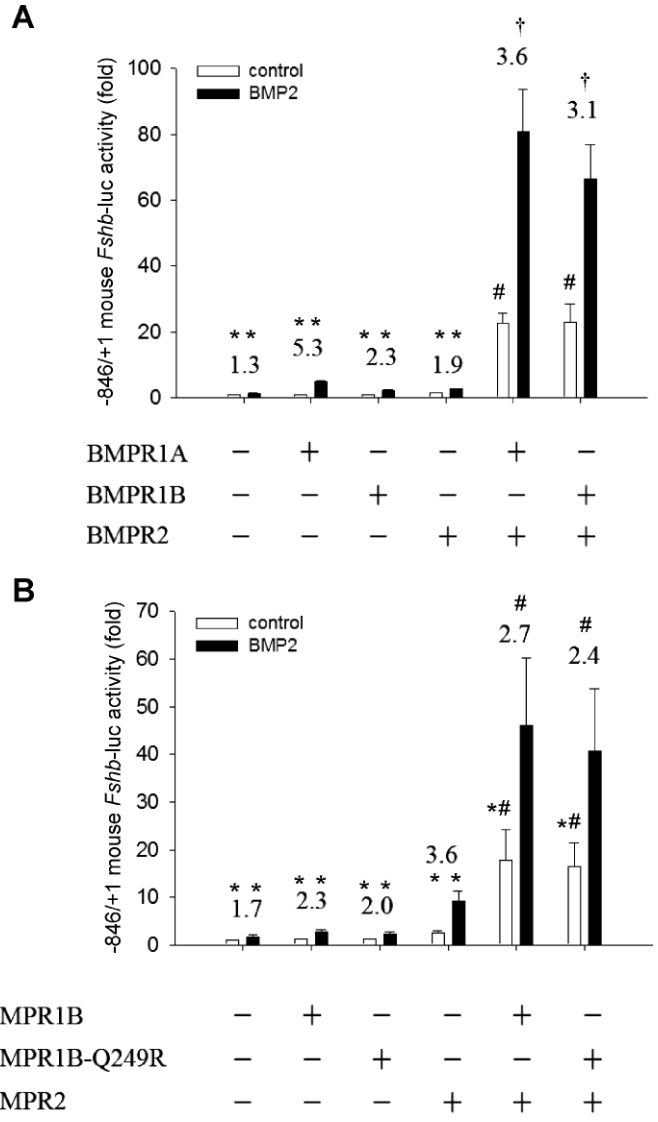

FIG. 1. **A**) LβT2 cells seeded in 24-well plates were transfected with −846/+1 mouse *Fshb*-luc and the indicated receptor expression vectors. Cells were then treated in duplicate with 25 ng/ml BMP2 in the presence of 10 μM SB431542 (to remove the effects of endogenous activin B signaling). **B**) LβT2 cells were transfected and treated as in **A** with the indicated receptor expression vectors. Cells were then treated in duplicate with 25 ng/ml BMP2 in the serum-free medium. In both panels, the data are the mean ± SEM of three independent experiments and are presented relative to the control group, in which no receptors or ligands were included. Bars with different symbols were statistically different, whereas those sharing symbols did not differ.

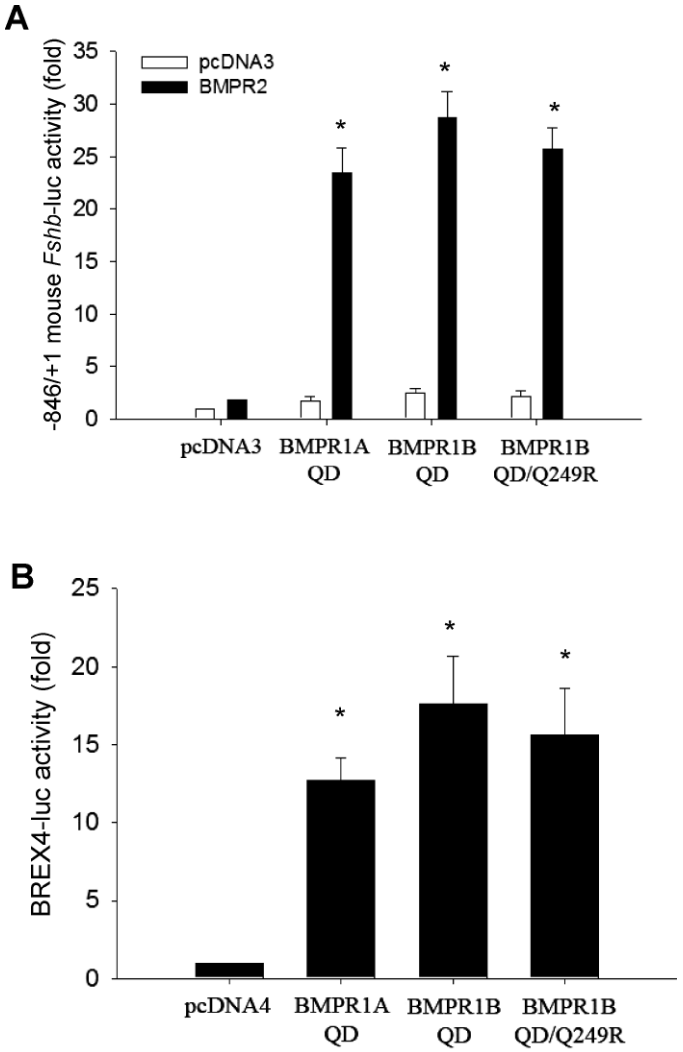

FIG. 2. **A**) LβT2 cells seeded in 24-well plates were transfected with −846/+1 mouse *Fshb*-luc and the indicated receptor expression vectors. **B**) HepG2 cells seeded in 24-well plates were transfected with BREX4-luc and the indicated receptor expression vectors. In both panels, cells were starved in serum-free medium for 24 h before analysis. The data are the mean ± SEM of three independent experiments and are presented relative to the control group, in which no receptors were transfected. QD indicates a Glu to Asp mutation at position 233 (BMPR1A) or 203 (BMPR1B). Bars with an asterisk are significantly different from bars without an asterisk but are not significantly different from one another.

control condition in each experiment. Differences between means were compared using one-way, two-way, or three-way ANOVAs, followed by post hoc pairwise comparison with Bonferroni or Tukey adjustment where appropriate (SYSTAT 10.2; Systat Software, Inc., Richmond, CA), as indicated in the figure legends. Significance was assessed relative to $P < 0.05$.

RESULTS

Transfected BMPR1A and BMPR1B Can Mediate BMP2 Signaling in Gonadotrophs

Results of previous transfection investigations in our laboratory suggested that ACVR1 might be the preferred type I receptor mediating the regulation of *Fshb* transcription by BMP2 [7]. Although we and others [18] observed *Bmpr1b* mRNA expression in the murine pituitary and LβT2 cells, we did not previously assess its role in BMP2 signaling. In addition, we discovered that the wild-type BMPR1A expression vector we had used previously [7] harbored an unwanted frameshift mutation that truncated the receptor within the kinase domain. This potentially invalidated the interpretation of our previous results [7] using this reagent. Therefore, we transfected LβT2 cells with the −846/+1 mouse *Fshb*-luc reporter and validated wild-type BMPR1A or BMPR1B receptor expression vectors alone or together with the type II receptor BMPR2. As observed previously with ACVR1 [7], either BMPR1A or BMPR1B with BMPR2 conferred heightened BMP2-independent and BMP2-dependent *Fshb* promoter activity (Fig. 1A). These effects were only observed when BMPR1A or BMPR1B was expressed in conjunction with

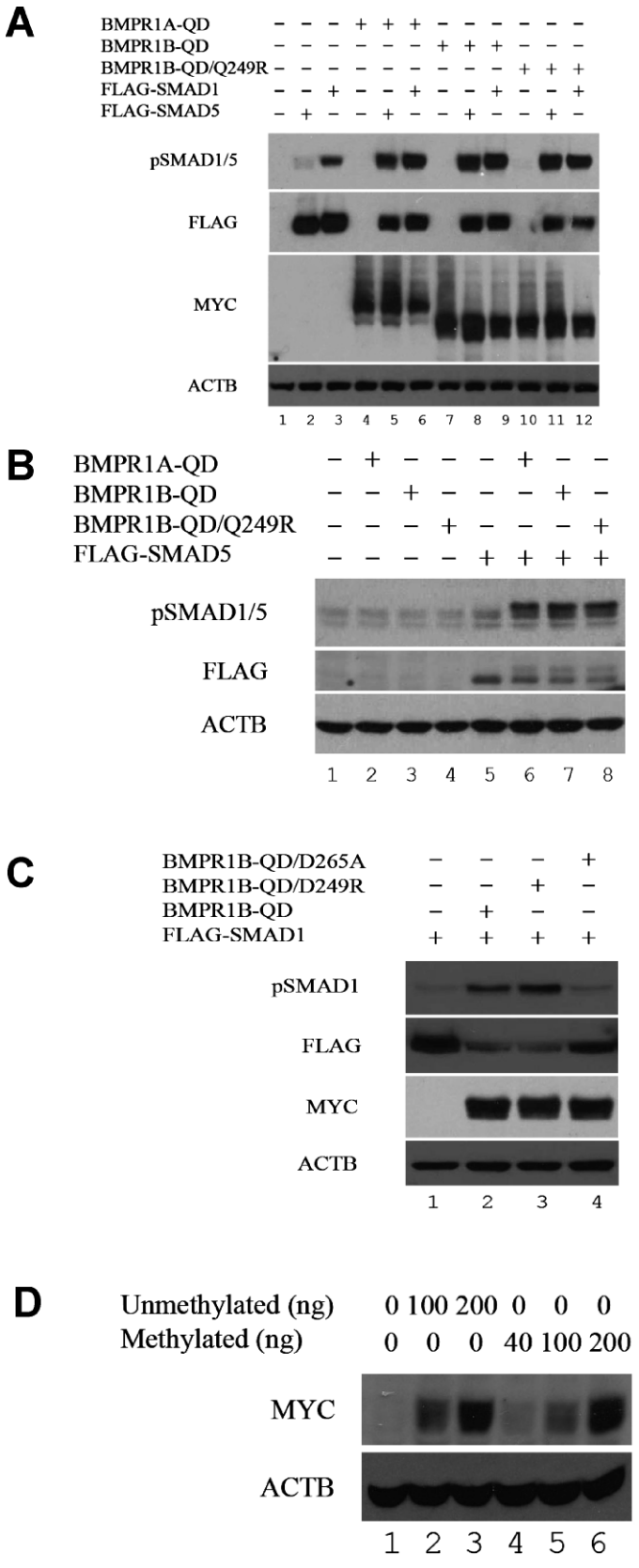

FIG. 3. **A**) CHO cells seeded in 6-well plates were transfected with the indicated constitutively active MYC-tagged type I receptors in conjunction with FLAG-tagged SMAD1 or SMAD5. Whole-cell protein lysates were subjected to Western blot analyses and were sequentially probed with pSMAD1/5/8, FLAG, MYC, and β-actin (ACTB) antibodies. **B**) LβT2 cells seeded in 6-well plates were transfected with the indicated constitutively active type I receptors in conjunction with FLAG-tagged SMAD5. Whole-cell lysates were subjected to Western blot analyses and were sequentially probed with pSMAD1/5/8, FLAG, and ACTB antibodies. **C**) CHO cells seeded in 10-cm plates were transfected with the indicated constitutively active MYC-tagged type I receptors in conjunction with FLAG-tagged SMAD1. Whole-cell lysates were subjected to Western blot analysis and were sequentially probed with pSMAD1/5/8, FLAG, MYC, and ACTB antibodies. **D**) CHO cells seeded in 6-well plates were transfected with the indicated amounts of methylated or unmethylated BMPR1B-QD/Q249R DNA constructs obtained directly from site-directed mutagenesis PCR reactions. Whole-cell protein lysates were subjected to Western blot analyses and were sequentially probed with MYC and ACTB antibodies.

BMPR2 and not when either was expressed alone. Similarly, constitutively active forms of BMPR1A (Q233D) and BMPR1B (Q203D) when expressed together with BMPR2, but not alone, stimulated *Fshb* promoter activity (Fig. 2A). Collectively, these results suggest that overexpressed ACVR1 (as shown previously [7]), BMPR1A, and BMPR1B can all regulate *Fshb* transcription in conjunction with BMPR2.

BMPR1B Harboring the Booroola Mutation Is Fully Functional in LβT2 Cells

A missense mutation, Q249R, was mapped to the kinase domain of BMPR1B in Booroola (*FecB*) sheep [23–25]. Given that *Bmpr1b* is expressed in the pituitary and may mediate BMP2 effects on *Fshb* (Figs. 1A and 2A), we investigated the effects of the BMPR1B-Q249R mutation on BMP2 signaling in gonadotroph cells. We introduced the mutation in the context of a murine BMPR1B expression vector. As already observed, expression of type I or type II receptors alone in LβT2 cells had no effect, whereas BMPR1B with BMPR2 up-regulated *Fshb* transcription, and this effect was further potentiated in the presence of BMP2 (Fig. 1B). Unexpectedly, the BMPR1B-Q249R mutant produced results equivalent to those of the wild-type BMPR1B receptor. Next, we examined potential functional differences between constitutively active forms of BMPR1B and BMPR1B-Q249R. The advantage of this approach is that it allowed us to examine functional changes in BMPR1B-Q249R that were independent of the particular ligand used in our experiments. As shown in Figure 2A, BMPR1A-QD, BMPR1B-QD, and BMPR1B-QD/Q249R all stimulated *Fshb* reporter activity when cotransfected with BMPR2 and did so to comparable extents.

To determine whether the results in LβT2 cells were cell specific, we assessed functionality of the constitutively active BMPR1A and BMPR1B receptors in HepG2 cells. Because *Fshb* reporters are inactive in nongonadotrophs, we used a validated BMP-responsive reporter, BREX4-luc [33]. We previously observed that constitutively active ACVR1 and BMPR1A regulated this reporter in these cells without the need for BMPR2 coexpression (data not shown). BMPR1A-QD, BMPR1B-QD, and BMPR1B-QD/Q249R all stimulated BREX4-luc activity in HepG2 cells and did so equivalently (Fig. 2B). Therefore, no obvious functional impairment in BMPR1B-Q249R was noted in two distinct cellular contexts.

BMPR1B-Q249R Can Stimulate SMAD1/5 Phosphorylation

In LβT2 and HepG2 cells, we failed to detect functional changes in BMPR1B-Q249R. One study [23] used molecular modeling to predict the effects of the Q249R mutation on receptor function, and the results suggested that the mutated receptor might more stably interact with the inhibitory protein

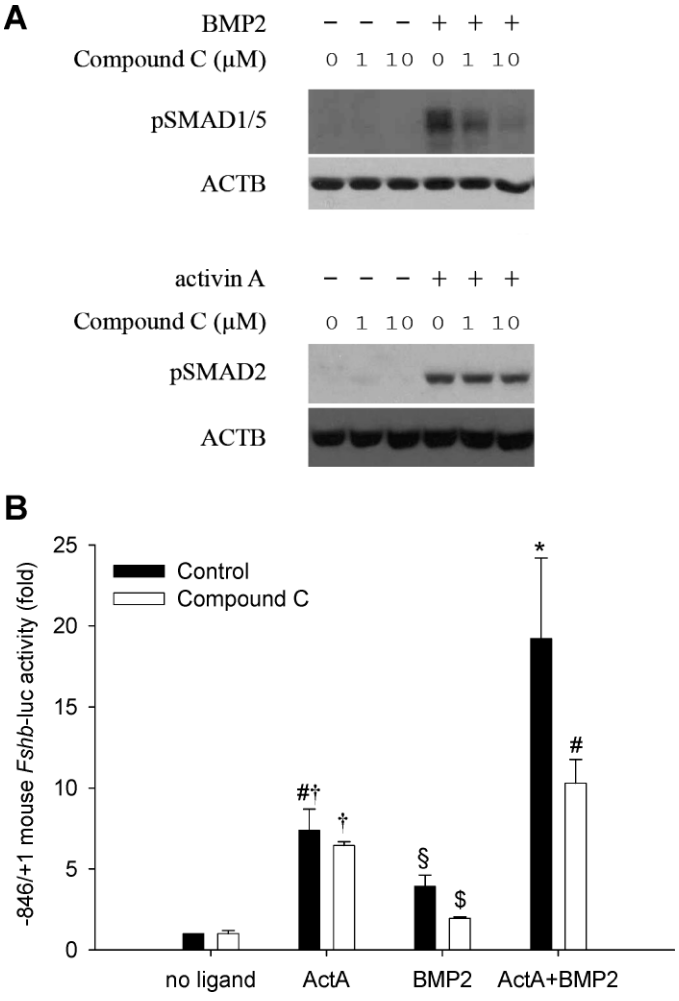

FIG. 4. **A**) LβT2 cells in 6-well plates were treated with 0, 1, or 10 μM compound C for 30 min, followed by treatment with 25 ng/ml BMP2 (top) or activin A (bottom) for 1 h. Whole-cell lysates were analyzed by Western blot for phospho-SMAD1/5/8 or phospho-SMAD2 as indicated. **B**) LβT2 cells were transfected with the indicated *Fshb* reporter and were treated with combinations of activin A (ActA) and BMP2 in the presence or absence of 10 μM compound C for 24 h. Data are the mean ± SEM of three independent experiments. Data were log transformed before analysis. Bars with different symbols were statistically different, whereas those sharing symbols did not differ.

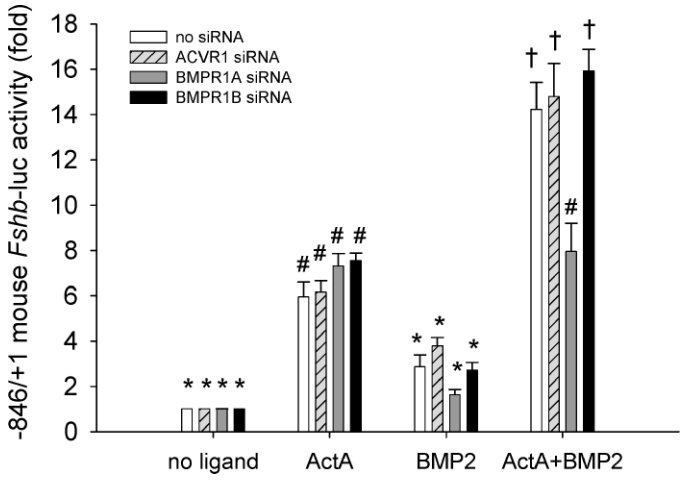

FIG. 5. LβT2 cells seeded in 24-well plates were transfected with −846/+1 mouse *Fshb*-luc and 5 nM of the indicated siRNAs for each of the three BMP type I receptors and were treated with 25 ng/ml BMP2 with or without 25 ng/ml activin A (ActA) in serum-free medium. The data are the mean ± SEM of three independent experiments and are presented relative to the control group, in which no siRNAs or ligands were included. Bars with different symbols were statistically different, whereas those sharing symbols did not differ.

FKBP12. This would be predicted to impair signaling by the receptor to its downstream effectors, including SMAD1 and SMAD5 [11, 13, 14]. Therefore, we examined the relative abilities of BMPR1A-QD, BMPR1B-QD, and BMPR1B-QD/Q249R to stimulate SMAD1 and SMAD5 phosphorylation. Use of constitutively active forms of the receptors obviated the need for exogenous ligand treatment. CHO cells were transfected with combinations of the indicated receptors and FLAG-SMAD1 or FLAG-SMAD5. Western blots using a phospho-SMAD1/5/8 antibody showed that all three receptors were equivalent in their abilities to stimulate SMAD1 and SMAD5 phosphorylation (Fig. 3A, top panel [compare lanes 5, 6, 8, 9, 11, and 12 vs. lanes 2 and 3]). Reprobing of the blots with FLAG (second panel) and MYC (third panel) antibodies confirmed equivalent expression of the SMADs and receptors, respectively. Similar results were observed in LβT2 cells (Fig. 3B). Thus, the BMPR1B-Q249R receptor seemed capable of stimulating SMAD1/5 phosphorylation to the same extent as the wild-type BMPR1B.

To confirm that point mutations can, in fact, impair BMPR1B function in these assays, we generated a novel mutation in BMPR1B, D265A. The aspartic acid at position 265 is only 16 amino acids C-terminal to Q249R and is located within the L45 loop of the receptor. This receptor subdomain has been implicated in SMAD activation by type I receptors [35–37]. The analogous mutation in TGFBR1 (also known as ALK5), D266A, has been reported to impair the ability of the receptor to stimulate SMAD2 phosphorylation [38]. Whereas BMPR1B-QD and BMPR1B-QD/Q249R stimulated SMAD1 phosphorylation, BMPR1B-QD/D265A was incapable of doing so (Fig. 3C). All three receptors were expressed at equivalent levels. Thus, our assays are able to detect impairments in receptor function.

BMPR1B-Q249R Is Expressed at Wild-Type Levels

Given that our analyses failed to show impairments in BMPR1B-Q249R function, we next examined whether the mutation affects receptor expression. Our initial analyses revealed equivalent expression of wild-type and Q249R forms of BMPR1B (Fig. 3, A–C, and data not shown). We noted that the mutation itself (CAG→CGG) introduces a novel CpG dinucleotide (underlined) that may be a substrate for DNA methylation. Although gene silencing is usually associated with methylation of cytosines in CpGs within promoter or enhancer regions, CpGs within coding regions might also be methylated and therefore have an effect on gene expression through their abilities to bind methyl DNA-binding proteins [39]. Although there was no apparent effect of the mutation on expression in transfected cells (Fig. 3, A–C), the DNA used was propagated in *Escherichia coli* and would not be methylated at this or other CpGs. Therefore, we introduced methylated cytosines on both strands of the BMPR1B-Q249R construct by site-directed mutagenesis using primers methylated specifically at the sites of interest. The same procedure was followed using identical primers that lacked methylcytosines. The resulting PCR products were then purified and transfected directly into CHO cells, and their relative

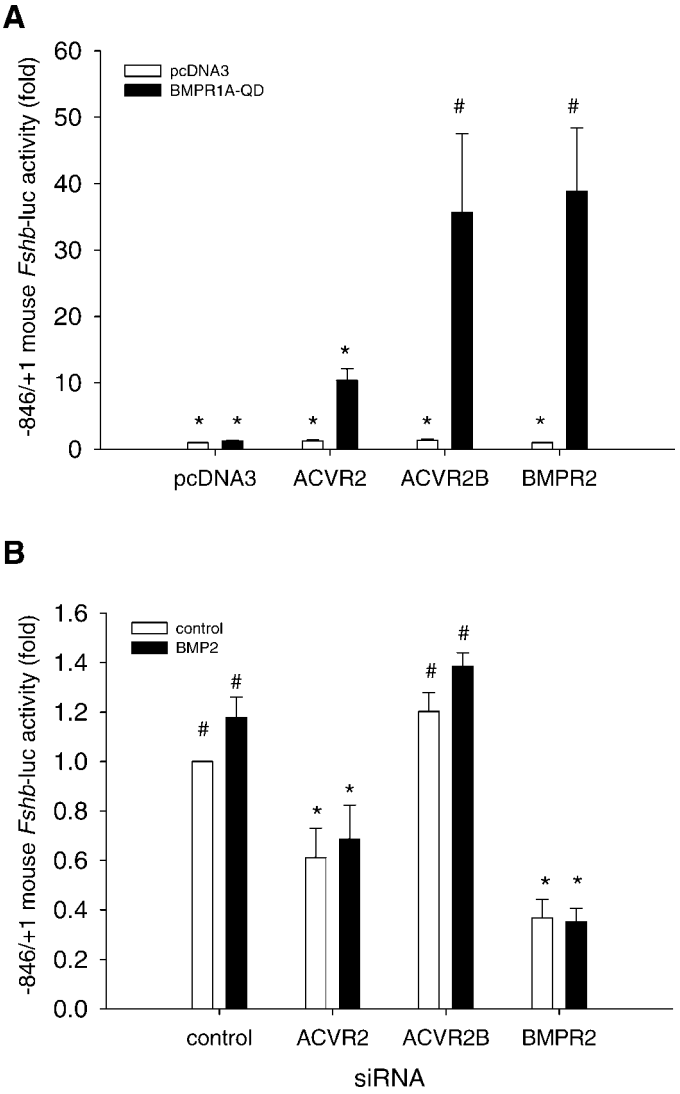

FIG. 6. **A**) LβT2 cells seeded in 24-well plates were transfected with −846/+1 mouse *Fshb*-luc and the indicated receptor expression vectors. Cells were starved in serum-free medium for 24 h before analysis. The data are the mean ± SEM of three independent experiments and are presented relative to the control group, in which no receptors were transfected. **B**) LβT2 cells were transfected with −846/+1 mouse *Fshb*-luc and 5 nM of the indicated siRNAs for each of the three BMP type II receptors and were treated with 25 ng/ml BMP2. The data are the mean ± SEM of three independent experiments and are presented relative to the control group, in which the control siRNA and no ligands were included. Bars with different symbols were statistically different, whereas those sharing symbols did not differ.

expression was measured by Western blot. Methylation of the amplified DNA was confirmed by bisulfite sequencing (data not shown). The methylated and unmethylated BMPR1B-Q249R constructs were expressed to equivalent extents (Fig. 3D); therefore, methylation at this site alone did not seem to affect receptor expression.

Endogenous BMPR1A Mediates BMP2 Signaling in Gonadotroph Cells

Although the data presented herein and previous findings [7] indicated that *Acvr1*, *Bmpr1a*, and *Bmpr1b* are expressed in LβT2 cells and can augment BMP2 actions when overexpressed in this cell line, the data did not definitely show whether BMP2 preferentially signals through one or more of these receptors. To confirm that ACVR1, BMPR1A, and/or BMPR1B is required for BMP2 signaling, we treated cells with compound C (also known as dorsomorphin), a small-molecule inhibitor of these three receptors [40]. We treated LβT2 cells with 1 μM or 10 μM compound C 30 min before treatment with 25 ng/ml BMP2 or activin A for 1 h. At 1 μM and 10 μM, the inhibitor significantly impaired BMP2-stimulated SMAD1/5 phosphorylation but did not affect activin A-stimulated SMAD2 phosphorylation (Fig. 4A). Increasing the concentration to 20 μM antagonized the BMP2 effect more significantly but also had a small inhibitory effect on activin A (data not shown). Therefore, in subsequent analyses, we used 10 μM compound C. We next transfected cells with a murine *Fshb* reporter and treated them with BMP2 with or without activin A in the presence or absence of compound C. The inhibitor significantly impaired the independent and synergistic actions of BMP2 on *Fshb* transcription but did not significantly alter the activin A response or basal reporter activity (Fig. 4B). These data suggested a role for endogenous ACVR1, BMPR1A, and/or BMPR1B in BMP2 signaling in LβT2 cells.

We next knocked down expression of ACVR1, BMPR1A, and/or BMPR1B by RNAi to determine which might be the preferred receptor in this system. LβT2 cells were transfected with −846/+1 mouse *Fshb*-luc and siRNAs for ACVR1, BMPR1A, or BMPR1B, and they were then treated with 25 ng/ml BMP2 with or without 25 ng/ml activin A. We observed the synergistic actions of BMP2 and activin A under control conditions and in the presence of the ACVR1 or BMPR1B siRNAs (Fig. 5). In contrast, the BMPR1A siRNA significantly inhibited the synergistic actions of BMP2 and activin A on *Fshb* reporter activity but did not impair the independent activin A response. The BMPR1A siRNA did not significantly diminish the independent BMP2 effect in the context of this analysis, although the trend was in this direction. These data suggested that BMPR1A is the preferred BMP2 type I receptor in LβT2 cells.

We confirmed the functionality and specificity of the siRNAs used in these experiments. LβT2 cells were transfected with epitope-tagged expression vectors for ACVR1, BMPR1A, or BMPR1B that were predicted to be sensitive or resistant to their respective siRNAs based on sequence match or mismatch. That is, we introduced mutations that rendered the expression constructs perfect matches (in rat ACVR1 and human BMPR1A) or created mismatches (in murine BMPR1B) relative to the murine siRNAs used in the experiment shown in Figure 5. In all cases, mutations altered the nucleotide but not the amino acid sequences. As shown in Supplemental Figure S1, the siRNAs specifically impaired expression of their sequence-matched ("sensitive") targets. The siRNAs directed against one receptor did not inhibit expression of the other receptors, and sequence-mismatched targets were resistant to their corresponding siRNAs. These data confirmed that the siRNA effects on receptor expression were sequence specific and did not reflect nonspecific or off-target effects.

Although the BMPR1A siRNA specifically impaired murine BMPR1A expression in LβT2 cells, we performed an additional control to show that decreases in *Fshb* reporter activity associated with the BMPR1A siRNA were attributable to receptor knockdown and not to some other off-target effect. We cotransfected LβT2 cells with −846/+1 mouse *Fshb*-luc and combinations of BMPR2 and siRNA-sensitive BMPR1A-QD or siRNA-resistant BMPR1A-QD along with control, BMPR1A, or BMPR1B siRNAs. The two forms of BMPR1A-QD equivalently stimulated reporter activity with BMPR2

(Supplemental Fig. S2). The BMPR1A, but not BMPR1B, siRNA inhibited the stimulatory effect of the sensitive, but not resistant, BMPR1A-QD expression vector, confirming that the BMPR1A siRNA effect was sequence specific.

Endogenous BMPR2 and ACVR2 Mediate BMP2 Signaling in Gonadotroph Cells

Finally, having established BMPR1A as the relevant endogenous type I receptor in LβT2 cells, we examined with which endogenous type II it cooperates to mediate BMP2 activity. BMP2 can bind BMPR2, ACVR2, and ACVR2B [41–43], and we showed previously that all three of these receptors are expressed in LβT2 cells and in adult murine pituitary [7]. We coexpressed BMPR1A-QD along with BMPR2, ACVR2, or ACVR2B expression vectors. None of the type II receptors had effects on their own, but all synergized with BMPR1A-QD to stimulate *Fshb* promoter activity (Fig. 6A). BMPR2 and ACVR2B had more pronounced effects than AVCR2. Next, we knocked down expression of the endogenous type II receptors using siRNAs. We cotransfected cells with the *Fshb* reporter and the indicated siRNAs, and we then treated them with 25 ng/ml BMP2 in the presence of the activin type I receptor inhibitor SB431542. Because we showed previously [7] that exogenous BMPs can synergize with endogenous activins in these cells, we needed to remove the potential confounding effects of activin signaling through ACVR2 or ACVR2B. Knockdown of BMPR2 or ACVR2 inhibited basal activity and the small (although not statistically significant) induction of *Fshb* transcription by BMP2 (Fig. 6B). The ACVR2B siRNA had no effect.

DISCUSSION

We reported previously that activin A and BMP2 synergistically regulate murine *Fshb* transcription [7]. We postulated that BMP2 might signal preferentially through the type I receptor ACVR1 to mediate its effects. This was based on the observation that transfection of wild-type ACVR1, but not BMPR1A, with the type II receptor BMPR2 stimulated promoter-reporter activity alone and in the presence of BMP2. In contrast, constitutively active forms of ACVR1 and BMPR1A both synergized with BMPR2 to stimulate *Fshb* transcription. We subsequently discovered that our presumptive wild-type BMPR1A expression vector possessed a frameshift mutation, which prematurely truncated the kinase domain of the receptor. When we repeated the analysis using a validated full-length receptor, we observed that BMPR1A functioned similarly to ACVR1 (Fig. 1A). A third BMP type I receptor, BMPR1B, is also expressed in LβT2 cells [7, 18] and can similarly act in synergy with BMPR2 to regulate *Fshb* transcription. These observations suggest that one or more type I receptors may mediate BMP signaling in gonadotroph cells. Indeed, inhibition of ACVR1, BMPR1A, and BMPR1B with compound C confirmed a role for at least one of these receptors in BMP2-regulated SMAD1/5 phosphorylation and *Fshb* reporter activity (Fig. 4).

To more definitely establish which receptor(s) might be most critical, we used siRNAs to deplete endogenous expression of ACVR1, BMPR1A, or BMPR1B. Although all of the siRNAs were effective in depleting expression of their targets in sequence-specific fashion (Supplemental Figs. S1 and S2), only BMPR1A knockdown blocked the synergistic actions of BMP2 and activin A on *Fshb* transcription (Fig. 5). The BMPR1A siRNA did not hinder activin A signaling by itself. These observations suggest that the effect of the BMPR1A siRNA is principally through antagonism of BMP2 signaling. BMP2 can signal through multiple type I and type II receptors [16, 44], and there is evidence for functional redundancy of the different receptors. For example, in the absence of BMPR2, BMP2 and BMP4 can signal through ACVR2 [16]. Herein, BMPR2 and ACVR2, but not ACVR2B, seemed to mediate the BMP2 response. Therefore, it is possible that ACVR1 and/or BMPR1B might compensate for the loss of BMPR1A, especially in light of the ability of these receptors to modulate *Fshb* transcription in overexpression experiments. However, the almost complete abrogation of BMP2-activin A synergism in the presence of the BMPR1A siRNA (Fig. 5) and the efficacy of ACVR1 and BMPR1B siRNAs in depleting their targets (Supplemental Fig. S1) suggest that neither ACVR1 nor BMPR1B compensates for the loss of BMPR1A in LβT2 cells, at least in these transient transfection assays. In light of these data and those with the type I receptor inhibitor (Fig. 4), we conclude that BMPR1A is the endogenous signal-propagating BMP2 receptor in these cells. Moreover, because overexpression of BMPR1B can potentiate the BMP2 response but knockdown of the endogenous receptor has no effect, we postulate that BMPR1B may be expressed at insufficient levels to propagate BMP2 signals in these cells.

Some Booroola (*FecB*) sheep that harbor a missense mutation (Q249R) in BMPR1B have increased FSH levels [22, 45, 46] in association with increased ovulation rates. Therefore, we hypothesized a priori that altered BMPR1B function might contribute to these phenotypes. The data presented herein failed to confirm this hypothesis on multiple levels. First (as already described), although it is expressed in gonadotroph cells, endogenous BMPR1B does not mediate BMP2 signaling. Second, the BMPR1B-Q249R receptor was functionally equivalent to the wild type in multiple assays. That is, the wild-type and mutant receptors stimulated two different reporters (*Fshb*-luc and BREx4-luc) in two different cell lines (LβT2 and HepG2) to equivalent extents (Figs. 1 and 2). Moreover, the receptors similarly stimulated SMAD1 and SMAD5 phosphorylation in CHO and LβT2 cells and were expressed at equivalent levels (Fig. 3, A–C). Most important, mutation of a nearby residue, D265A, completely abrogated BMPR1B-regulated SMAD1 phosphorylation (Fig. 3C), demonstrating the sensitivity of our experimental approach.

We also examined whether the Q249R mutation might affect receptor expression, perhaps through DNA methylation (Fig. 3D). However, the methylated and unmethylated Q249R receptors were expressed at equivalent levels, which is consistent with a previous study [23] showing equivalent *Bmpr1b* mRNA levels in wild-type and Booroola sheep ovaries.

In conclusion, the data presented herein show that BMP2 regulates murine *Fshb* subunit transcription independently and synergistically with activin A by signaling through the type I receptor BMPR1A and the type II receptors BMPR2 and ACVR2. Although ACVR1 and BMPR1B are expressed in LβT2 cells and in murine pituitary and both can act with BMPR2 to regulate *Fshb* promoter activity in overexpression analyses, neither seems necessary for BMP2 action, nor does either compensate for the loss of BMPR1A. We further show that the Q249R mutation observed in BMPR1B of Booroola sheep does not alter the ability of the receptor to stimulate SMAD1/5 phosphorylation or to activate target gene transcription in different cellular contexts. Future investigations will be required to confirm a role for BMPR1A in FSH regulation in vivo and to determine the nature of altered BMPR1B function in Booroola (*FecB*) sheep.

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REFERENCES

1. Dupont J, McNeilly J, Vaiman A, Canepa S, Combarnous Y, Taragnat C. Activin signaling pathways in ovine pituitary and LbetaT2 gonadotrope cells. Biol Reprod 2003; 68:1877–1887.
2. Bernard DJ. Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells. Mol Endocrinol 2004; 18:606–623.
3. Suszko MI, Balkin DM, Chen Y, Woodruff TK. Smad3 mediates activin-induced transcription of follicle-stimulating hormone beta-subunit gene. Mol Endocrinol 2005; 19:1849–1858.
4. Lamba P, Santos MM, Philips DP, Bernard DJ. Acute regulation of murine follicle-stimulating hormone beta subunit transcription by activin A. J Mol Endocrinol 2006; 36:201–220.
5. Bernard DJ, Lee KB, Santos MM. Activin B can signal through both ALK4 and ALK7 in gonadotrope cells. Reprod Biol Endocrinol 2006; 4: e52.
6. Harrison CA, Gray PC, Vale WW, Robertson DM. Antagonists of activin signaling: mechanisms and potential biological applications. Trends Endocrinol Metab 2005; 16:73–78.
7. Lee KB, Khivansara V, Santos MM, Lamba P, Yuen T, Sealfon SC, Bernard DJ. Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone beta subunit transcription. J Mol Endocrinol 2007; 38:315–330.
8. Huang HJ, Wu JC, Su P, Zhirnov O, Miller WL. A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology 2001; 142:2275–2283.
9. Otsuka F, Shimasaki S. A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 2002; 143:4938–4941.
10. Nicol L, Faure MO, McNeilly JR, Fontaine J, Taragnat C, McNeilly AS. Bone morphogenetic protein-4 interacts with activin and GnRH to modulate gonadotrophin secretion in LbetaT2 gonadotrophs. J Endocrinol 2008; 196:497–507.
11. Miyazono K. Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins. Bone 1999; 25:91–93.
12. Allendorph GP, Vale WW, Choe S. Structure of the ternary signaling complex of a TGF-beta superfamily member. Proc Natl Acad Sci U S A 2006; 103:7643–7648.
13. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells 2002; 7: 1191–1204.
14. Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev 2005; 19:2783–2810.
15. Suszko MI, Lo DJ, Suh H, Camper SA, Woodruff TK. Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. Mol Endocrinol 2003; 17:318–332.
16. Yu PB, Beppu H, Kawai N, Li E, Bloch KD. Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. J Biol Chem 2005; 280:24443–24450.
17. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 2004; 25:72–101.
18. Suszko MI, Woodruff TK. Cell-specificity of transforming growth factor-beta response is dictated by receptor bioavailability. J Mol Endocrinol 2006; 36:591–600.
19. Davis GH, Montgomery GW, Allison AJ, Kelly RW, Bray AR. Segregation of a major gene influencing fecundity in progeny of Booroola sheep. N Z J Agric Res 1982; 25:525–529.
20. Piper LR. The single gene inheritance of the high litter size of the Booroola Merino. In: Land RB, Robinson DW (eds.), Genetics of Reproduction in Sheep. London: Butterworths; 1985:115–125.
21. Monget P, Fabre S, Mulsant P, Lecerf F, Elsen JM, Mazerbourg S, Pisselet C, Monniaux D. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domest Anim Endocrinol 2002; 23:139–154.
22. McNatty KP, Hudson N, Henderson KM, Gibb M, Morrison L, Ball K, Smith P. Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate. J Reprod Fertil 1987; 80:577–588.
23. Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, et al. Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. Proc Natl Acad Sci U S A 2001; 98:5104–5109.
24. Souza CJ, MacDougall C, Campbell BK, McNeilly AS, Baird DT. The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. J Endocrinol 2001; 169:R1–R6.
25. Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP, Montgomery GW. Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. Biol Reprod 2001; 64: 1225–1235.
26. Campbell BK, Baird DT, Souza CJ, Webb R. The FecB (Booroola) gene acts at the ovary: in vivo evidence. Reproduction 2003; 126:101–111.
27. Fabre S, Pierre A, Pisselet C, Mulsant P, Lecerf F, Pohl J, Monget P, Monniaux D. The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality. J Endocrinol 2003; 177:435–444.
28. Young JM, Juengel JL, Dodds KG, Laird M, Dearden PK, McNeilly AS, McNatty KP, Wilson T. The activin receptor-like kinase 6 Booroola mutation enhances suppressive effects of bone morphogenetic protein 2 (BMP2), BMP4, BMP6 and growth and differentiation factor-9 on FSH release from ovine primary pituitary cell cultures. J Endocrinol 2008; 196: 251–261.
29. Faure MO, Nicol L, Fabre S, Fontaine J, Mohoric N, McNeilly A, Taragnat C. BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. J Endocrinol 2005; 186:109–121.
30. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994; 22:2990–2997.
31. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992; 89:1827–1831.
32. Liu F, Ventura F, Doody J, Massague J. Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. Mol Cell Biol 1995; 15:3479–3486.
33. Hata A, Seoane J, Lagna G, Montalvo E, Hemmati-Brivanlou A, Massague J. OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. Cell 2000; 100:229–240.
34. Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 2002; 62:65–74.
35. Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engstrom U, Heldin CH, Funa K, ten Dijke P. The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. FEBS Lett 1998; 434:83–87.
36. Chen YG, Hata A, Lo RS, Wotton D, Shi Y, Pavletich N, Massague J. Determinants of specificity in TGF-beta signal transduction. Genes Dev 1998; 12:2144–2152.
37. Feng XH, Derynck R. A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. EMBO J 1997; 16:3912–3923.
38. Itoh S, Thorikay M, Kowanetz M, Moustakas A, Itoh F, Heldin CH, ten Dijke P. Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. J Biol Chem 2003; 278:3751–3761.
39. Clouaire T, Stancheva I. Methyl-CpG binding proteins: specialized transcriptional repressors or structural components of chromatin? Cell Mol Life Sci 2008; 65:1509–1522.
40. Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD, Peterson RT. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 2008; 4:33–41.
41. Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin CH, Miyazono K. Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 1995; 130:217–226.
42. Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K. Cloning and characterization of a

human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A 1995; 92:7632–7636.
43. Kawabata M, Chytil A, Moses HL. Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-beta receptor. J Biol Chem 1995; 270:5625–5630.
44. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 1994; 269:16985–16988.
45. Bindon BM. Reproductive biology of the Booroola Merino sheep. Aust J Biol Sci 1984; 37:163–189.
46. Robertson DM, Ellis S, Foulds LM, Findlay JK, Bindon BM. Pituitary gonadotrophins in Booroola and control Merino sheep. J Reprod Fertil 1984; 71:189–197.

Endocrinology

Bone Morphogenetic Protein 2 Acts via Inhibitor of DNA Binding Proteins to Synergistically Regulate Follicle-Stimulating Hormone ^{2} Transcription with Activin A

Catherine C. Ho and Daniel J. Bernard

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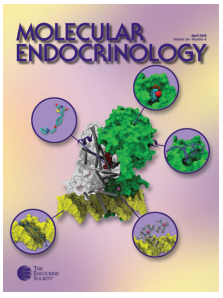

Bone Morphogenetic Protein 2 Acts via Inhibitor of DNA Binding Proteins to Synergistically Regulate Follicle-Stimulating Hormone β Transcription with Activin A

Catherine C. Ho and Daniel J. Bernard

Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1Y6

We recently reported that bone morphogenetic proteins (BMPs) 2 and 4 can stimulate FSHβ-subunit (*Fshb*) transcription alone and in synergy with activins. We further showed that BMP2 signals via the BMP type IA receptor (or activin receptor-like kinase 3) to mediate its effects. However, the intracellular mechanisms through which BMP2 regulates *Fshb* are unknown. In the current study, we used cDNA microarray analyses (and validation by real-time quantitative RT-PCR) to identify BMP2 target genes in the murine gonadotrope cell line, LβT2. Short-interfering RNA-mediated knockdown, overexpression, and coimmunoprecipitation experiments were used to examine the potential functional roles of selected gene products. Quantitative RT-PCR analysis largely confirmed the results of the array analyses, and inhibitors of DNA binding 1, 2, and 3 (Id1, Id2, and Id3) were selected for functional analyses. Knockdown of endogenous *Id2* or *Id3,* but not *Id1,* diminished the synergistic effects of BMP2 and activin A on *Fshb* transcription. Overexpression of Id1, Id2, or Id3 alone had no effect, but all three potentiated activin A or mothers against decapentaplegic homolog (SMAD)3 induction of *Fshb* transcription. Though the precise mechanism through which Ids produce their effects are not yet known, we observed physical interactions between Id1, Id2, or Id3 and SMAD3. Collectively, the data suggest that BMP2 synergistically regulates *Fshb* transcription with activins, at least in part, through the combined actions of Ids 2 or 3 and SMAD3. **(*Endocrinology* 151: 3445–3453, 2010)**

The pituitary gonadotropins, FSH and LH, play essential roles in reproductive physiology. Perturbations in either the expression or activity of these hormones or their receptors lead to infertility in females and oligospermia or infertility in males (1–4). Both hormones are secreted from gonadotropes of the anterior pituitary and stimulate gonadal steroidogenesis and gametogenesis. The gonadotropins are heterodimeric glycoproteins (α/β), sharing a common α-subunit and unique β-subunits. The latter determine both rates of mature hormone synthesis and biological specificity. FSH and LH are regulated by gonadotropin-releasing hormone 1, secreted from the hypothalamus, as well as gonadal sex steroids. FSH synthesis is also regulated by the activins and inhibins, members of the transforming growth factor β (TGFβ) superfamily (5–9). Recently, other TGFβ ligands, the bone morphogenetic proteins (BMPs), were shown to stimulate murine *Fshb* transcription alone and in synergy with activins *in vitro* (10–13). In contrast, BMP4 was shown to block the stimulatory effect of activins on FSH secretion from sheep pituitary cultures (14), suggesting potential interspecies variation in BMP action.

Several BMP subtypes are expressed in adult murine pituitary and in immortalized gonadotropes, LβT2 (10–12). However, the *in vivo* role, if any, for these proteins in FSH regulation has not yet been established. Although BMP6 and BMP7 are endogenously expressed in LβT2 cells, they regulate *Fshb* transcription with low potency. In contrast, BMP2 and BMP4, which are expressed at low

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Abbreviations: ALK, Activin receptor-like kinase; BMP, bone morphogenetic protein; bHLH, basic HLH; HA, hemagglutinin; HLH, helix-loop-helix; Id, inhibitor of DNA binding; IP, immunoprecipitation; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; RNAi, RNA interference; siRNA, short-interfering RNA; SMAD, mothers against decapentaplegic homolog; TGFβ, transforming growth factor β; WCE, whole-cell protein extract.

levels in these cells, are able to stimulate *Fshb* transcription with greater potency (10). Because BMP2 and BMP4 are highly expressed in the adult murine pituitary (presumably by other cell types), they may act as paracrine regulators of gonadotrope function.

BMP2 and BMP4 are less potent than activins in their induction of *Fshb* transcription. However, BMPs and activins have strong synergistic actions (10–13). Therefore, for *Fshb* regulation, BMPs may be more important for their cooperative than independent actions. In neither case, however, do we have a clear mechanistic understanding of BMP's effects. We previously demonstrated that BMP2 signals via the BMP type IA receptor [also known as activin receptor-like kinase (ALK)3] to stimulate *Fshb* transcription in LβT2 cells (15). Overexpression approaches also implicated the signaling protein, mothers against decapentaplegic homolog (SMAD)8, in BMP2-stimulated *Fshb* expression. However, a role for the endogenous SMAD8 has not yet been established nor do we know how overexpressed SMAD8, directly or indirectly, produces its effects. To gain greater insight into how BMP2 may regulate *Fshb* in gonadotropes, we used cDNA microarrays to identify BMP2 target genes in LβT2 cells. Under our experimental conditions, a relatively limited number of genes were regulated by BMP2. However, follow-up analyses implicate the inhibitors of DNA binding 2 and 3 (Id2 and Id3) in BMP2/activin A synergistic induction of murine *Fshb* transcription.

Materials and Methods

Reagents

Human recombinant activin A and BMP2 were purchased from R&D Systems (Minneapolis, MN). Gentamycin, 1× PBS, and DMEM with 4.5 g/liter glucose, L-glutamine, and sodium pyruvate were purchased from Wisent (St. Bruno, Quebec, Canada). F-12/DMEM with 2.5 mM L-glutamine, 15 mM HEPES buffer, and 1.2 g/liter sodium bicarbonate was purchased from HyClone Laboratories (South Logan, UT). Random primers, Moloney murine leukemia virus-reverse transcriptase, RNasin, and deoxynucleotide triphosphates, and 1× passive lysis buffer was from Promega (Madison, WI). Protease inhibitor tablets (Complete Mini) were purchased from Roche (Mississauga, Ontario, Canada). EZview Red ANTI-FLAG M2 Affinity Gel (catalog no. F2426), SB431542, mouse monoclonal β-actin (no. A5441), mouse monoclonal hemagglutinin (HA) (no. H9658), and rabbit monoclonal FLAG (no. F3165) antibodies were from Sigma (St. Louis, MO). Horseradish peroxidase conjugated secondary antibodies were from Bio-Rad (Hercules, CA) and enhanced chemiluminescence Plus reagent was from GE Healthcare (Piscataway, NJ). Short-interfering RNAs (siRNAs) were purchased from Dharmacon (Lafayette, CO): Control (catalog no. D-001210-05), IDB1 (ID1, catalog no. D-040701-17), IDB2 (ID2, catalog no. D-060495-02), IDB3 no. 2 (ID3, catalog no. D-046495-02), and IDB3 no. 3 (ID3, catalog no. D-046495-03). Lipofectamine/Plus, Lipofectamine 2000, TRIzol Reagent, and SYBRgreen Supermix for quantitative PCR (qPCR) were from Invitrogen (Carlsbad, CA). In solution, MG132 proteasome inhibitor was purchased from Calbiochem (San Diego, CA) (catalog no. 474791).

Constructs

The murine and porcine *Fshb* promoter-reporter constructs were described previously (16, 17). The SMAD3-responsive $CAGA_{12}$-luc reporter was described previously (18) and by Dennler *et al.* (19). HA-tagged murine Id1, Id2, and Id3 expression constructs were generously provided by Nacksung Kim (20). Human FLAG-SMAD1, human FLAG-SMAD3, and murine FLAG-SMAD4 were provided by T. Woodruff (Northwestern University, Chicago, IL). Human FLAG-SMAD2 and human FLAG-SMAD3 were provided by E. Robertson (University of Oxford, Oxford, UK). Murine FLAG-SMAD5 and FLAG-SMAD6 were provided by T. Watanabe (Tokyo University, Tokyo, Japan). Murine FLAG-SMAD7 and rat myc-SMAD8 were provided by C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The rat FLAG-SMAD8 construct was generated in-house by PCR using myc-SMAD8 as template. Human FLAG SMAD3 N-terminal only (N), N-terminal plus linker (NL), linker plus C-terminal (LC), and C-terminal only (C) were purchased from Addgene (Cambridge, MA).

Cell cultures and transfections

Immortalized murine gonadotrope LβT2 cells were provided by P. Mellon (University of California, San Diego, CA) and were cultured in 10% fetal bovine serum/DMEM and 4 μg/ml gentamycin as described previously (21). For gene array experiments and quantitative RT-PCR (qRT-PCR) analyses (see details below), LβT2 cells cultured in 10-cm dishes for approximately 48 h were washed with serum-free DMEM and then treated for 24 h with 2 nM (50 ng/ml) BMP2 in DMEM. The ALK4/5/7 inhibitor SB431542 (22) was included (final concentration 10 μM) to block the effects of endogenous activin B. Cells were washed with 1× PBS and total RNA extracted with TRIzol (Invitrogen) following the manufacturer's instructions.

For luciferase assays, cells were plated in 24-well plates (2.5×10^5 cells per well) or in 48-well plates (0.8×10^5 cells per well) approximately 36 h before transfection. Cells were transfected with 450 or 225 ng of the reporter/well, respectively, using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). In Id1, Id2, or Id3 overexpression experiments, LβT2 cells cultured in 24- or 48-well plates were transfected with 50 or 25 ng of expression plasmid per well, respectively. In both cases, cells were treated with 1 nM activin A and lysates collected 24 h after treatment. In SMAD3 and Id1, Id2, or Id3 overexpression experiments, LβT2 cells cultured in 24- or 48-well plates were transfected with 100 or 50 ng of the SMAD3 expression plasmid per well. Cells were changed to serum-free media and lysates collected 24 h later. In RNA interference (RNAi) experiments, siRNAs in 1× siRNA buffer [20 mM KCl, 6 mM HEPES (pH7.5), and 0.2 mM $MgCl_2$] were transfected at a final concentration of 5 nM. Resulting data were normalized to cells transfected with the control siRNA. Twenty-four hours after transfection, cells were washed in 1× PBS and treated with 1 nM (25 ng/ml) activin A and/or BMP2 in DMEM or with DMEM alone (no ligand control) for 24 h.

CHO cells were obtained from P. Morris (Population Council, New York, NY) and cultured in F-12/DMEM containing

10% fetal bovine serum and 4 μg/ml gentamycin. CHO cells in six-well plates were transfected when 70–80% confluent using Lipofectamine/Plus and 100–250 ng of the indicated Id expression vectors and 10 nM of the indicated control or Id siRNAs for 6 h and then changed to growth media. Cell protein lysates were harvested the day after for use in immunoblot analyses (see below). CHO cells grown in 10-cm dishes were transfected in the same manner as in the six-well plates using Lipofectamine/Plus and 4 μg of the indicated HA-tagged Id and FLAG-tagged SMAD expression vectors. Cell protein lysates were harvested the day after for use in immunoprecipitation (IP) analyses (see below). In one experiment (as indicated), 10 μM MG132 in growth media was included 5 h before harvest.

Gene array

Affymetrix GeneChips (430 version 2.0; Affymetrix, Santa Clara, CA) were used to identify BMP2 target genes in LβT2 cells. Total RNA was collected from cells treated as described above and submitted to The Rockefeller University Genomics Resource Center for processing (The Rockefeller University, New York, NY). Two sets of raw chip data (n = 2) from the microarrays were analyzed using FlexArray (version 1.3 from GenomeQuébec) first by background correction then by data normalization. The average difference for each gene between treated and untreated cells was calculated and the fold change in gene expression determined. Supplemental Fig. 1A published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org shows the mean of two different arrays represented as a scatter plot. Data points that stray furthest from the identity line represent genes that showed the greatest fold change in response to BMP2 treatment. Data were then analyzed using EB Wright & Simon statistical analysis, and the results presented as a Volcano Plot (Supplemental Fig. 1B). Cluster analysis of the microarray data was performed with the DAVID online functional annotation tool (available at david.abcc.ncifcrf.gov/). The protein products of the regulated genes can be broadly categorized into functional groups implicated in TGFβ signaling, transcriptional regulation, cardiac development, muscle contraction, negative regulation of cellular metabolic process, and one gene encoded a protein of unknown function.

RT and qRT-PCR

RT was performed on 1–2 μg of total RNA as previously described (21). qRT-PCR was performed on the resulting cDNA using the SYBRgreen Supermix following manufacturer's instructions with the Corbett Rotorgene 6000 qPCR machine (Corbett Life Science, San Francisco, CA). Data represent the mean of three independent experiments (n = 1 per experiment). Expression of target genes was normalized relative to ribosomal protein L19 (*Rpl19*) in the same sample. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method (23, 24), and the data presented relative to the no ligand control. Sequences of the qPCR primers for the various target genes are shown in Supplemental Table 1.

Luciferase assays

Cells were washed with 1× PBS and lysed in 1× passive lysis buffer. Luciferase assays were performed on an Orion II microplate luminometer (Berthold Detection Systems, Oak Ridge, TN) using standard reagents. All treatments were performed in triplicate as described in the text or figure legends. Data are represented as means of means from three or more independent experiments.

IP

Cells were washed with 1× PBS and whole-cell protein extracts (WCE) prepared with lysis buffer [50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100] and centrifuged at 10,000 rpm for 15 min at 4 C to remove insoluble material. WCEs were subjected to IP using EZview Red ANTI-FLAG M2 Affinity Gel following the manufacturer's instructions. In short, WCEs were incubated with the affinity gel on a rotating platform overnight at 4 C to allow binding of FLAG-SMAD3 to the Anti-FLAG affinity gel. The affinity gel was then incubated in 1× FLAG peptide (catalog no. F4799) solution on a rotating platform for 45 min at 4 C to elute gel-bound proteins. The eluted proteins were then analyzed by immunoblot (see below).

Immunoblotting

Cells were washed with 1× PBS and WCEs prepared in 1× radioimmunoprecipitation assay buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 0.01 M sodium phosphate (pH 6.8), 2 mM EDTA, 50 mM sodium fluoride, and Complete Mini Protease Inhibitor Cocktail Tablets] and centrifuged at 13,000 rpm for 0.5 h at 4 C to remove insoluble material. WCEs (or eluted proteins from IPs) were subjected to immunoblot analyses as previously described (21). Briefly, equivalent amounts of protein were separated by SDS-PAGE and transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH). Blots were probed with the indicated antibodies using standard techniques.

Data analysis

Reporter assay and qPCR data from three replicate experiments were highly similar. Therefore, means of treatment replicates within each experiment were calculated to generate a single value per treatment per experiment. The data from replicate (three or more) experiments were then used for statistical analyses. Data are presented as fold change from the control condition (no ligand and/or transfection with empty vector alone). Differences between means of untransformed or log-transformed data were compared using one-, two-, or three-way ANOVA followed by *post hoc* pair-wise comparison with Bonferroni or Tukey adjustment where appropriate (Systat 10.2; Systat, Richmond, CA). Significance was assessed relative to $P < 0.05$.

Results

Identification of BMP2-regulated genes by microarray analysis

cDNA microarrays were used to identify BMP2 target genes in LβT2 cells. These cells synthesize activin B (10, 25). Therefore, to remove effects derived from synergistic actions of exogenous BMP2 and endogenous activin B, we blocked the latter with the small molecule inhibitor, SB431542 (22). BMP2 (2 nM for 24 h) stimulated an increase in mRNA levels of 18 genes (*Id1*, *Id2*, *Id3*, *Asgr1*,

TABLE 1. BMP2-regulated genes in LβT2 cells

Gene	qRT-PCR (fold change)	Microarray (fold change)	*P* (microarray)
Inhibitor of DNA binding 3 (*Id3*)	10.1	15.6	5.5E-08
Gastrokine 3 (*Gkn3*)[a]	–	4.8	2.1E-05
Inhibitor of DNA binding 1 (*Id1*)	9.1	4.7	5.8E-07
Inhibitor of DNA binding 2 (*Id2*)	7.7	3.8	6.5E-07
Troponin I, Cardiac (*Tnni3*)[a]	–	3.7	1.0E-06
TBC1 domain family, member 10A (*Tbc1d10a*)	9.2	3.7	2.7E-06
MAD homolog 6 (*Smad6*)	8.7	3.6	6.3E-07
Antimicrobial peptide RYA3 (*Rya3*)[a]	–	3.5	5.5E-07
Ellis van creveld syndrome 2 homolog (*Evc2*)[a]	–	3.3	9.2E-07
Cholinergic receptor, nicotinic, α polypeptide 2 (*Chrna2*)[a]	–	3.0	1.4E-06
Troponin T1, skeletal, slow (*Tnnt1*)[a]	–	2.4	1.1E-05
ATPase, C2+ transporting, type2C, member 2 (*Atp2c2*)	1.2	2.4	1.8E-05
Regulator of G-protein signaling 6 (*Rgs6*)	25.9	2.3	2.3E-04
Epoxide Hydrolase 2 (*Ephx2*)[a]	–	2.3	7.6E-06
Kallikrein 7 (*Klk7*)[a]	–	2.2	2.0E-05
Homeo box gene expressed in ES cells (*Hesx1*)	3.1	2.1	1.2E-05
Asialoglycoprotein receptor 1 (*Asgr1*)[a]	–	2.1	1.8E-04
Growth Differentiation factor 9 (*Gdf9*)	3.7	2.1	1.6E-05
Thyroglobulin (*Tg*)[a]	–	0.5	2.9E-05
Serine/Threonine kinase 25 (*Stk25*)	0.8	0.5	9.0E-04
Matrilin 1 cartilage matrix protein 1 (*Matn1*)[a]	–	0.5	1.5E-05
Calcium/calmodulin-dependent protein kinase IV (*Camk4*)	0.7	0.5	4.8E-03
Crystallin, μ (*Crym*)[a]	–	0.5	4.3E-05
Carboxypeptidase a1 (*Cpa1*)[a]	–	0.5	1.8E-05
Calbindin-28K (*Calb1*)	0.4	0.4	6.7E-05

E, Exponent; –, not measured.

[a] Genes not analyzed by qRT-PCR.

Atp2c2, *Chrna2*, *Ephx2*, *Evc2*, *Gdf9*, *Gkn3*, *Hesx1*, *Klk7*, *Rgs6*, *Rya3*, *Smad6*, *Tbc1d10a*, *Tnni3*, and *Tnnt1*) (Supplemental Fig. 1 and Table 1), with the magnitude of change ranging from 2- to 15-fold. BMP2 also down-regulated seven transcripts (*Calb1*, *Camk4*, *Cpa1*, *Crym*, *Matn1*, *Stk25*, and *Tg*) (Supplemental Fig. 1 and Table 1) by greater than 2-fold.

qRT-PCR was used to validate the results from the microarray analysis. LβT2 cells were treated with BMP2 and SB431542 in the same manner as for the microarray analysis. RNA was collected after 24 h, and relative expression levels of nine up-regulated (Fig. 1A) and three down-regulated genes (Fig. 1B) were examined by qRT-PCR. These 12 genes were selected based on our interest in their putative functions. The changes in gene expression observed in qRT-PCR mirrored qualitatively those observed with the microarray, although the overall magnitude of the response differed between the two methods. One gene that differed on the array, *Atp2c2*, showed no change in response to BMP2 treatment by qRT-PCR. The correct identity of the PCR products was verified by restriction digest or direct sequencing (data not shown). Although *Fshb* expression in LβT2 cells (BMP2 treated or untreated) was below the threshold of detection on the microarray, we observed a BMP2-stimulated increase by qRT-PCR (data not shown). However, because the *Fshb* mRNA expression level in cells not treated with BMP2 (*i.e.* control cells) was at or below the detection limit of the qRT-PCR assay, it was difficult to accurately assess the precise fold induction.

Depletion of endogenous *Id2* or *Id3* inhibits both activin A and activin A/BMP2 regulation of *Fshb* transcription

Id1, *Id2*, and *Id3* were among the genes most up-regulated by BMP2 (Supplemental Fig. 1 and Fig. 1A and Table 1). Id proteins have previously been implicated as effectors of BMP signaling in a variety of cellular systems (26–33). To determine a potential role for endogenous Id proteins in BMP2 regulation of *Fshb* transcription, we used siRNAs to deplete their expression in LβT2 cells. Cells were transfected with a murine *Fshb* promoter-reporter and the indicated siRNAs. After 24 h, cells were then treated with 1 nM BMP2 ± 1 nM activin A. Knockdown of *Id2* or *Id3*, but not *Id1*, diminished both activin A and activinA/BMP2-stimulated reporter activity (Fig. 2). To confirm the efficacy of the siRNAs, we examined the effects of the siRNAs on ID1/2/3 protein expression. Under our assay conditions, transfection efficiency of LβT2 cells is insufficient to obtain an accurate measure of the extent of RNAi-mediated knockdown of mRNA/protein expression on a per cell basis (21). Therefore, to validate the siRNAs, we overexpressed murine HA-Id1/2/3

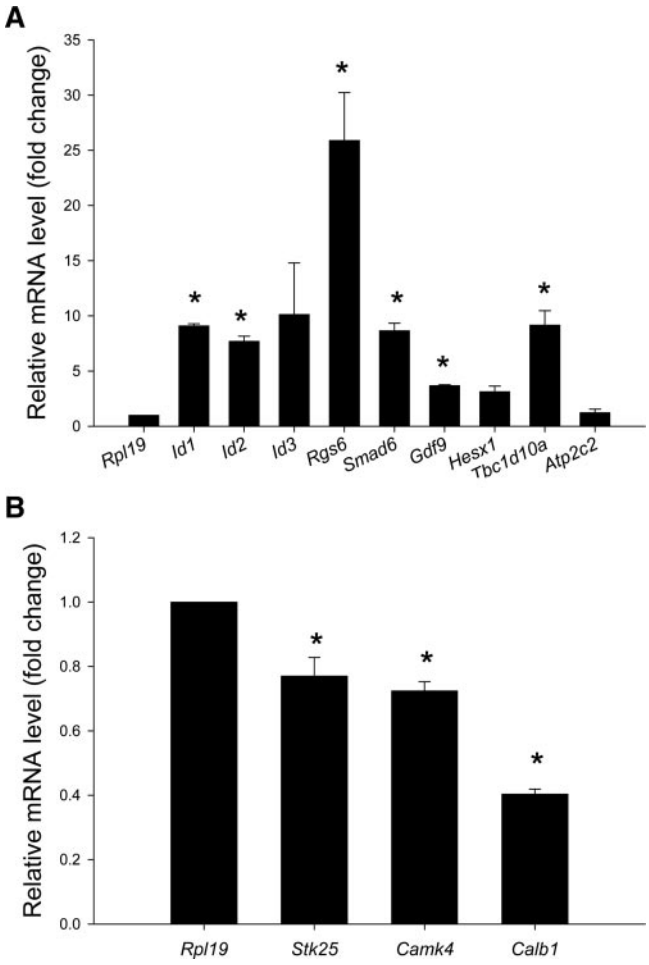

FIG. 1. LβT2 cells seeded in 10-cm dishes were treated with vehicle or 50 ng/ml BMP2 in SF-DMEM containing 10 μM SB431542 for 24 h. RNA was extracted, and changes in expression of the indicated genes were measured by qRT-PCR. Data reflect the mean (+SEM) from three independent experiments and are normalized to the housekeeping gene, *Rpl19*, and presented as fold change in mRNA expression relative to untreated cells. *Bars with asterisks* were statistically different from 1, Bonferroni adjustment ($P < 0.05$). Up- and down-regulated genes are shown in A and B, respectively.

constructs in CHO cells in the presence or absence of the *Id1/2/3* siRNAs (Supplemental Fig. 2). Each siRNA potently inhibited protein expression of its target. Collectively, these data suggest that Id2 and Id3 may mediate BMP2's synergistic but not independent effects on *Fshb* transcription.

Id proteins potentiate the stimulatory effect of activin A on *Fshb* transcription

The data in Fig. 2 suggested that Id protein expression under both untreated and BMP2-stimulated conditions modulates activin A induction of *Fshb* transcription. To determine whether Ids can substitute for BMP2 to regulate *Fshb* transcription, we transfected LβT2 cells with a murine *Fshb* promoter-reporter along with Id1, Id2, or Id3 expression constructs. Cells were then treated with 1 nM activin A for 24 h. Id1, Id2, or Id3 expression alone did not

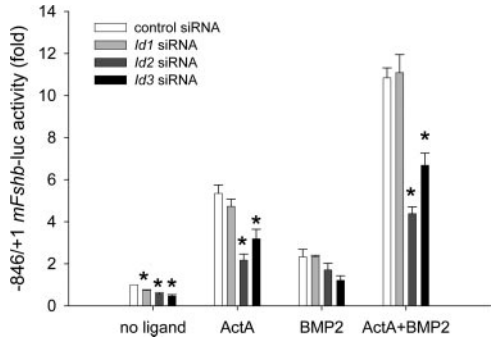

FIG. 2. LβT2 cells seeded in 24-well plates were transfected with murine −846/+1 *Fshb*-luc and 5 nM of the control siRNA or siRNAs for the indicated *Id*s and treated with 25 ng/ml activin A (ActA), BMP2, or both for 24 h in serum-free medium. The data reflect the mean (+SEM) luciferase activity from three independent experiments and are presented relative to the control group, in which control siRNA was transfected, but no ligands were included. *Bars with asterisks* are statistically different from their respective control with the same treatment conditions, Bonferroni adjustment ($P < 0.05$).

significantly alter *Fshb* reporter activity (Fig. 3). However, expression of Id1, Id2, or Id3 significantly potentiated the stimulatory effect of activin A. These data suggest that Id regulation of *Fshb* transcription is activin dependent.

Id proteins functionally interact with SMAD3 to stimulate *Fshb* transcription

Activins stimulate *Fshb* transcription, at least in part, via SMAD3 in LβT2 cells (21, 34, 35). We therefore examined whether Ids cooperate with SMAD3 to regulate *Fshb*. We transfected LβT2 cells with a murine *Fshb* promoter-reporter along with Id1, Id2, or Id3 expression constructs in the presence or absence of a SMAD3 expression construct. As expected, SMAD3 alone strongly up-regulated murine *Fshb* transcription, whereas Id1, 2, or 3 alone did not (Fig. 4A). However, transfection of Ids in combination with SMAD3 significantly potentiated the stimu-

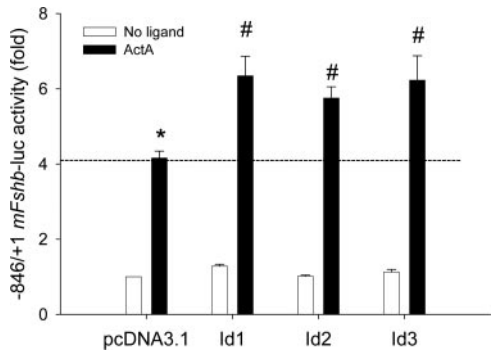

FIG. 3. LβT2 cells seeded in 24- or 48-well plates were transfected with −846/+1 *Fshb*-luc along with 50 or 25 ng/well, respectively, of the indicated Id1, Id2, or Id3 expression constructs. Cells were then treated with 25 ng/ml activin A (ActA) for 24 h. Data are the means (+SEM) of five independent experiments and are presented relative to untreated cells transfected with the empty expression vector. *Bars with different symbols* were statistically different, whereas *bars sharing symbols* did not differ, Bonferroni adjustment ($P < 0.05$). m, Murine.

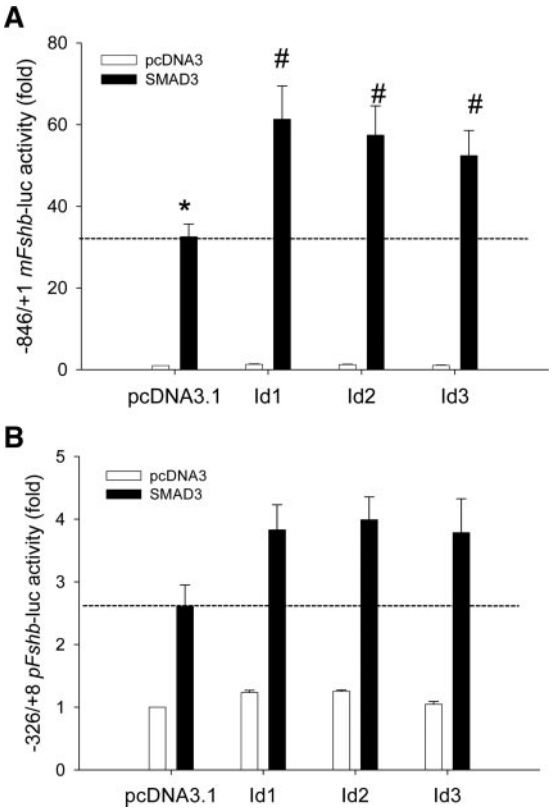

FIG. 4. A, LβT2 cells seeded in 24- or 48-well plates were transfected with −846/+1 *Fshb*-luc along with 50 or 25 ng/well, respectively, of the indicated Id1, Id2, and/or Id3 expression constructs and 100 or 50 ng/well, respectively, of the SMAD3 expression construct. Cells were cultured in serum-free media 24 h before harvest. Data are the means (+SEM) of 11 independent experiments and are presented relative to cells transfected with the empty expression vectors. Statistical analysis was done with log-transformed data with Tukey adjustment. *Bars with different symbols* were statistically different, whereas *bars sharing symbols* did not differ ($P < 0.05$). m, Murine. B, LβT2 cells seeded in 24-well plates were transfected with a porcine −326/+8 *Fshb*-luc reporter construct along with 50 ng/well of the indicated Id1, Id2, and/or Id3 expression constructs and 100 ng/well of the SMAD3 expression construct. Cells were cultured in serum-free media 24 h before harvest. Data are the means (+SEM) of three independent experiments and are presented relative to cells transfected with the empty expression vectors.

latory effect of SMAD3. The same pattern of results was observed with a porcine *Fshb* promoter-reporter (Fig. 4B). Although there was a significant main effect of Id overexpression, the SMAD3 × Id interaction was not significant ($P = 0.358$), precluding pair-wise comparisons. In contrast, Ids failed to potentiate SMAD3 induction of the SMAD3/4-responsive reporter $CAGA_{12}$-luc (data not shown). These data suggested that the combined actions of Id proteins and SMAD3 are promoter specific.

Id proteins physically interact with SMAD3

We next asked whether the functional interaction between Ids and SMAD3 might reflect physical interactions between the proteins. FLAG-SMAD3 and HA-Id1, HA-Id2, or HA-Id3 were cotransfected in CHO cells. Whole-cell lysates were harvested, SMAD3-containing complexes were immunoprecipitated with FLAG affinity gel, and interacting Ids assessed by immunoblot with an HA antibody. All three Id proteins were pulled down with FLAG-SMAD3 (Fig. 5A), suggesting that the proteins are part of the same complex.

Next, we assessed the SMAD3 subdomain(s) mediating the interaction with Ids. SMAD3 has three functional domains: the N-terminal MH1 domain (N), a linker domain (L), and the C-terminal MH2 domain (C) (36). Given that all three Ids physically interacted with SMAD3, we used Id2 as a reference to determine the interacting domain in SMAD3. CHO cells were cotransfected with HA-Id2 and the FLAG-tagged full-length SMAD3, SMAD3N, SMAD3NL, SMAD3LC, or SMAD3C expression vectors. Whole-cell lysates were collected and subjected to IP/immunoblot analysis as in Fig. 5A. The data show a preferential interaction between Id2 and SMAD3 MH2 domain (Fig. 5B). The identities of the multiple bands in the SMAD3LC and SMAD3C lanes were not determined. However, the original paper describing these constructs reported the same banding pattern (37).

Id2 selectively interacts with SMAD3 and SMAD8

To determine whether the interaction between Id and SMAD proteins is general or specific, we examined Id2's interaction with all eight mammalian SMAD proteins. CHO cells were cotransfected with HA-Id2 and FLAG-SMAD1, FLAG-SMAD2, FLAG-SMAD3, FLAG-SMAD4, FLAG-SMAD5, FLAG-SMAD6, FLAG-SMAD7, or FLAG-SMAD8 expression vectors. As in the foregoing analysis, whole-cell lysates were subjected to IP/immunoblot analysis. Although all SMADs were expressed to roughly equivalent levels, Id2 interacted exclusively with SMAD3 and SMAD8 (Fig. 6).

Discussion

We previously demonstrated that BMP2 potentiates activin A-induction of *Fshb* transcription in immortalized murine gonadotropes (10). In addition, we identified ALK3 (BMP type IA receptor) as the preferred type I receptor mediating BMP2 actions (15). In the current study, we further dissected the downstream pathway(s) through which BMP2 may act to modulate activin A-induction of *Fshb* transcription. We used cDNA microarrays as a search tool for candidate regulators. To our surprise, relatively few genes were BMP2-regulated. This may be attributable to the inclusion of the ALK4/5/7 inhibitor, SB431542, to remove the confounding effects of endogenous activin B. Moreover, we only examined a single time

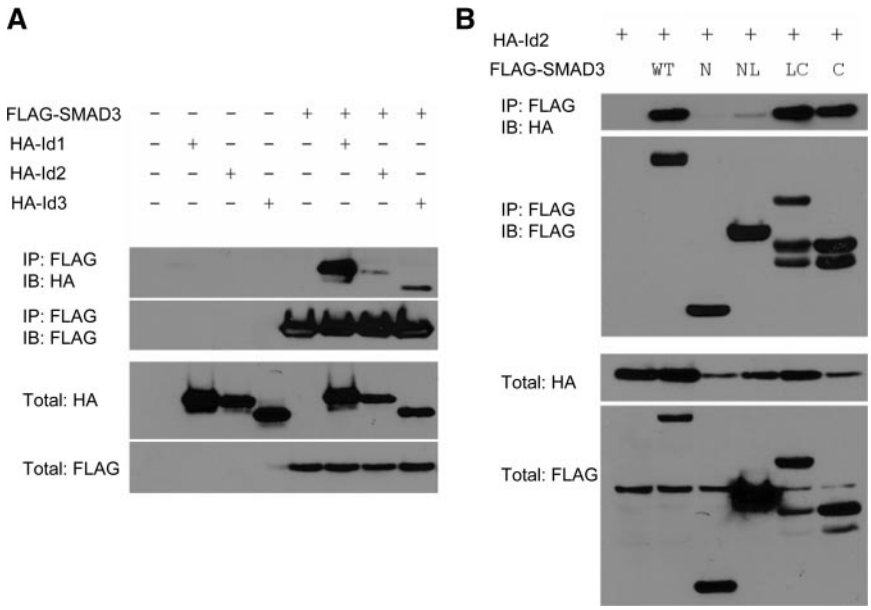

FIG. 5. A, CHO cells seeded in 10-cm dishes were transfected with 4 μg of HA-Id1, HA-Id2, or HA-Id3 and 4 μg of FLAG-SMAD3 expression vector. Whole-cell lysates were collected for FLAG-IP analysis. IP proteins were subjected to immunoblot (IB) analysis with FLAG and HA antibodies. B, CHO cells seeded in 10-cm dishes were transfected with 4 μg of HA-Id2 and 4 μg of FLAG-SMAD3 (wild type), FLAG-SMAD3N (N), FLAG-SMAD3NL (NL), FLAG-SMAD3LC (LC), or FLAG-SMAD3C (C) expression vectors. Cells were treated with 10 μM MG132 5 h before harvest. IP/immunoblots were performed as in B.

point and dose of BMP2 treatment. Nonetheless, known BMP-response genes *Id1*, *Id2*, *Id3*, and *Smad6* were among the up-regulated transcripts (26–33, 38), validating our results. Importantly, the qRT-PCR analysis largely confirmed the results of the array analyses. Because Id proteins have been implicated as effectors of BMP signaling in other contexts (28), we explored their potential roles in *Fshb* transcription.

We suppressed endogenous Id1, Id2, and Id3 expression with siRNAs. Depletion of Id2 or Id3 attenuated both activin A and activin A plus BMP2-induction of *Fshb* transcription but did not affect the BMP2 response. In contrast, Id1 knockdown was without effect. These data suggest that BMP2 may synergize with activins, at least in part, via up-regulating Id2 and/or Id3 production, but that BMP2's independent effects on *Fshb* transcription do not require stimulated Id expression. Consistent with this hypothesis, Id1, Id2, or Id3 overexpression potentiated the activin A response, while having no effect on their own. Because Id2 or Id3 knockdown also antagonized the independent activin A response, basal levels of Id2 or Id3 may play previously unappreciated roles in activin-induced *Fshb* transcription. It is interesting to note that Id1 overexpression, but not knockdown, potentiated the activin A response. We do not currently know the cause of these apparently discrepant results. One possibility is that neither basal nor BMP2-stimulated Id1 protein levels are sufficiently high to modulate activin A's effects. Alternatively, Id2 or Id3 might compensate for the absence of Id1. Data from knockout models are consistent with this latter possibility (28, 39–42).

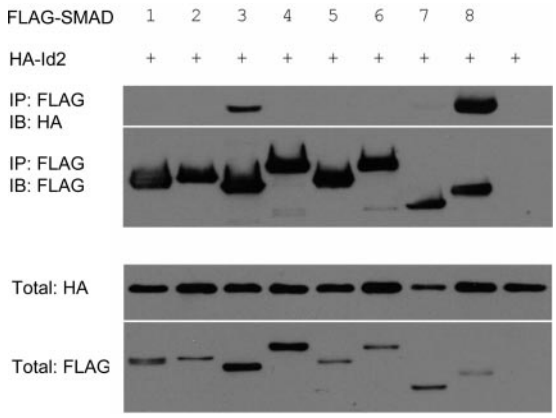

FIG. 6. CHO cells seeded in 10-cm dishes were transfected with 4 μg of HA-Id2 and 4 μg of FLAG-SMAD1, FLAG-SMAD2, FLAG-SMAD3, FLAG-SMAD4, FLAG-SMAD5, FLAG-SMAD6, FLAG-SMAD7, or FLAG-SMAD8 expression vectors. IP/immunoblots were performed as in Fig. 5.

Activins regulate *Fshb* via SMAD proteins, in particular SMAD3 (21, 34, 35). Indeed, SMAD3 overexpression is sufficient to stimulate *Fshb* transcription in LβT2 cells. We therefore asked whether Ids modulate SMAD3 activity. SMAD3 potently stimulated murine *Fshb* promoter activity, and this effect was potentiated by all three Id proteins, which again had no effect on their own. These data are consistent with our previous observation that BMP2 potentiates the effects of overexpressed SMAD3 (10). We examined Id modulation of SMAD3 induction of two additional reporters: porcine *Fshb*-luc and $CAGA_{12}$-luc. As with the murine reporter, the porcine *Fshb* promoter is SMAD3 responsive and synergistically regulated by activin A and BMP2 (10, 17). The pattern of results with the porcine promoter was highly similar to that with the murine promoter. We observed significant main effects of both SMAD3 and Id overexpression. Unlike the case with the murine promoter, however, the SMAD3 $\times$ Id interaction was not statistically significant, suggesting that the combined actions of the proteins were additive rather than synergistic. This could reflect interspecies differences or perhaps limitations of the analyses. We prefer the latter explanation. That is, with additional replications of the experiment (and more statistical power), the interaction might have been significant. Indeed, one should note that the Ids when expressed alone did not modify re-

porter activity. Instead, their actions were only observed in the presence of coexpressed SMAD3. In contrast, Ids failed to potentiate or modify SMAD3 induction of the SMAD3/4-responsive reporter, $CAGA_{12}$-luc (data not shown) (18, 19). The data therefore suggest that SMAD3/Id cooperativity (either synergism or additivity) reflects a promoter-specific rather than a general phenomenon (*i.e.* that Ids to not generally modify SMAD3 activity).

We next asked whether a physical interaction might underlie the cooperative actions of Ids and SMAD3. By co-IP, we observed interactions between all three Ids examined and SMAD3. We further mapped the interaction to the SMAD3 MH2 domain. To our knowledge, this is the first demonstration of SMAD3-Id interactions. Ids are members of the helix-loop-helix (HLH) family of transcription factors. Interestingly, SMAD3 was similarly shown to interact with myogenic differentiation antigen 1, a basic HLH (bHLH) transcription factor, via its MH2 domain (43). Thus, although we did not map the interaction domain in the Ids, the observation that all three Ids and myogenic differentiation antigen 1 interact with SMAD3 suggests that the common HLH domain likely mediates the interaction. Given the conservation of the MH2 domain across the eight SMAD family members in mammals, we were surprised to see that SMAD8 was the only other SMAD to interact with Id2. The basis for this specificity is not yet known. However, it may be important to note that we previously observed that overexpressed SMAD8, but not other BMP-regulated SMADs (1 and 5), potently stimulates murine *Fshb* promoter activity in BMP2-treated LβT2 cells (10).

Although SMAD3 and Id proteins can physically interact, how this might lead to their functional interaction is not yet known. We examined whether coexpression of Ids impacted SMAD3 nuclear localization but observed no effect (data not shown). SMAD binding to DNA, which is weak on its own, is enhanced through protein-protein interaction. That is, SMADs can interact with high(er) affinity DNA binding cofactors (36, 44–52). It is therefore tempting to speculate that Ids might increase SMAD3 affinity for the *Fshb* promoter. However, there is no evidence the Ids bind directly to DNA. Another possibility is that a bHLH family member might interact with SMAD3, inhibiting its activity at the *Fshb* promoter (53). Ids might then compete for binding to SMAD3, relieving this repression. Future investigations aimed at identifying bHLH proteins interacting with SMAD3 in LβT2 cells will provide an important first step in testing this hypothesis.

In summary, we used global gene expression profiling to identify BMP2-regulated genes in LβT2 cells. Among the up-regulated transcripts were the Ids which are known BMP-response genes in other cellular systems (26–33, 38). By RNAi-mediated knockdown, we demonstrate that Id2 and Id3 contribute to activin A and activinA/BMP2 regulation of *Fshb* expression. How the Ids produce their effects are not entirely clear. However, overexpression and co-IP data suggest that a physical interaction between Ids and SMAD3 may form part of the underlying mechanism.

Acknowledgments

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Address all correspondence and requests for reprints to: Daniel J. Bernard, Ph.D., Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada H3G 1Y6. E-mail: daniel.bernard@mcgill.ca.

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References

1. **Conway GS** 1996 Clinical manifestations of genetic defects affecting gonadotrophins and their receptors. Clin Endocrinol 45:657–663
2. **Chan WY** 1998 Molecular genetic, biochemical, and clinical implications of gonadotropin receptor mutations. Mol Genet Metab 63:75–84
3. **Kumar TR** 2005 What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? Reproduction 130:293–302
4. **Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV** 2001 Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 15:184–200
5. **Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R** 1986 Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321:779–782
6. **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
7. **Welt C, Sidis Y, Keutmann H, Schneyer A** 2002 Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. Exp Biol Med 227:724–752
8. **Welt CK** 2004 Regulation and function of inhibins in the normal menstrual cycle. Semin Reprod Med 22:187–193
9. **Winters SJ, Moore JP** 2004 Intra-pituitary regulation of gonadotrophs in male rodents and primates. Reproduction 128:13–23
10. **Lee KB, Khivansara V, Santos MM, Lamba P, Yuen T, Sealfon SC, Bernard DJ** 2007 Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone β subunit transcription. J Mol Endocrinol 38:315–330
11. **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
12. **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

13. **Nicol L, Faure MO, McNeilly JR, Fontaine J, Taragnat C, McNeilly AS** 2008 Bone morphogenetic protein-4 interacts with activin and GnRH to modulate gonadotrophin secretion in LβT2 gonadotrophs. J Endocrinol 196:497–507
14. **Faure MO, Nicol L, Fabre S, Fontaine J, Mohoric N, McNeilly A, Taragnat C** 2005 BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. J Endocrinol 186:109–121
15. **Ho CC, Bernard DJ** 2009 Bone morphogenetic protein 2 signals via BMPR1A to regulate murine follicle-stimulating hormone β subunit transcription. Biol Reprod 81:133–141
16. **Lamba P, Santos MM, Philips DP, Bernard DJ** 2006 Acute regulation of murine follicle-stimulating hormone β subunit transcription by activin A. J Mol Endocrinol 36:201–220
17. **Lamba P, Fortin J, Tran S, Wang Y, Bernard DJ** 2009 A novel role for the forkhead transcription factor FOXL2 in activin A-regulated follicle-stimulating hormone β subunit transcription. Mol Endocrinol 23:1001–1013
18. **Filyak Y, Filyak O, Souchelnytskyi S, Stoika R** 2008 Doxorubicin inhibits TGF-β signaling in human lung carcinoma A549 cells. Eur J Pharmacol 590:67–73
19. **Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM** 1998 Direct binding of Smad3 and Smad4 to critical TGF β-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J 17:3091–3100
20. **Lee J, Kim K, Kim JH, Jin HM, Choi HK, Lee SH, Kook H, Kim KK, Yokota Y, Lee SY, Choi Y, Kim N** 2006 Id helix-loop-helix proteins negatively regulate TRANCE-mediated osteoclast differentiation. Blood 107:2686–2693
21. **Bernard DJ** 2004 Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone β subunit in mouse gonadotrope cells. Mol Endocrinol 18:606–623
22. **Inman GJ, Nicolás FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS** 2002 SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 62:65–74
23. **VanGuilder HD, Vrana KE, Freeman WM** 2008 Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44:619–626
24. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ Δ C(T)) method. Methods 25:402–408
25. **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
26. **Locklin RM, Riggs BL, Hicok KC, Horton HF, Byrne MC, Khosla S** 2001 Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology. J Bone Miner Res 16:2192–2204
27. **Samanta J, Kessler JA** 2004 Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development 131:4131–4142
28. **Miyazono K, Miyazawa K** 2002 Id: a target of BMP signaling. Sci STKE 2002:pe40
29. **Coppé JP, Smith AP, Desprez PY** 2003 Id proteins in epithelial cells. Exp Cell Res 285:131–145
30. **Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T** 1994 Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766
31. **Abe J** 2006 Bone morphogenetic protein (BMP) family, SMAD signaling and Id helix-loop-helix proteins in the vasculature: the continuous mystery of BMPs pleotropic effects. J Mol Cell Cardiol 41:4–7
32. **Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, Luu HH, Park JK, Li X, Luo J, Montag AG, Haydon RC, He TC** 2004 Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. J Biol Chem 279:32941–32949
33. **Kowanetz M, Valcourt U, Bergström R, Heldin CH, Moustakas A** 2004 Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor β and bone morphogenetic protein. Mol Cell Biol 24:4241–4254
34. **Dupont J, McNeilly J, Vaiman A, Canepa S, Combarnous Y, Taragnat C** 2003 Activin signaling pathways in ovine pituitary and LβT2 gonadotrope cells. Biol Reprod 68:1877–1887
35. **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
36. **Massagué J, Seoane J, Wotton D** 2005 Smad transcription factors. Genes Dev 19:2783–2810
37. **Zhang Y, Musci T, Derynck R** 1997 The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. Curr Biol 7:270–276
38. **Takase M, Imamura T, Sampath TK, Takeda K, Ichijo H, Miyazono K, Kawabata M** 1998 Induction of Smad6 mRNA by bone morphogenetic proteins. Biochem Biophys Res Commun 244:26–29
39. **Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R** 1999 Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature 401:670–677
40. **Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y** 1997 High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. Mol Cell Biol 17:7317–7327
41. **Kim JK, Takeuchi M, Yokota Y** 2004 Impairment of intestinal intraepithelial lymphocytes in Id2 deficient mice. Gut 53:480–486
42. **Rivera RR, Johns CP, Quan J, Johnson RS, Murre C** 2000 Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. Immunity 12:17–26
43. **Liu D, Black BL, Derynck R** 2001 TGF-β inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. Genes Dev 15:2950–2966
44. **Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li JM, Wang XF** 1999 Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor β. Mol Cell Biol 19:1821–1830
45. **Chen X, Rubock MJ, Whitman M** 1996 A transcriptional partner for MAD proteins in TGF-β signalling. Nature 383:691–696
46. **Chen X, Weisberg E, Fridmacher V, Watanabe M, Naco G, Whitman M** 1997 Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature 389:85–89
47. **Zhang Y, Feng XH, Derynck R** 1998 Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature 394:909–913
48. **Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, Stavnezer J, Kawabata M, Miyazono K, Ito Y** 1999 Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Cα promoter. J Biol Chem 274:31577–31582
49. **Hata A, Seoane J, Lagna G, Montalvo E, Hemmati-Brivanlou A, Massagué J** 2000 OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. Cell 100:229–240
50. **Chen CR, Kang Y, Siegel PM, Massagué J** 2002 E2F4/5 and p107 as Smad cofactors linking the TGFβ receptor to c-myc repression. Cell 110:19–32
51. **Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C, Piccolo S** 2003 Links between tumor suppressors: p53 is required for TGF-β gene responses by cooperating with Smads. Cell 113:301–314
52. **Seoane J, Le HV, Shen L, Anderson SA, Massagué J** 2004 Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell 117:211–223
53. **Grinberg AV, Kerppola T** 2003 Both Max and TFE3 cooperate with Smad proteins to bind the plasminogen activator inhibitor-1 promoter, but they have opposite effects on transcriptional activity. J Biol Chem 278:11227–11236

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Mechanisms of bone morphogenetic protein 2 (BMP2) stimulated inhibitor of DNA binding 3 (*Id3*) transcription☆

Catherine C. Ho[a], Xiang Zhou[a], Yuji Mishina[b], Daniel J. Bernard[a,*]

[a] Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada H3G 1Y6
[b] University of Michigan School of Dentistry, Dept. of Biologic and Materials Sciences, 4222A Dental, 1011 N. University Ave., Ann Arbor, MI 48109-1078, USA

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ABSTRACT

Bone morphogenetic protein 2 (BMP2) stimulates expression of the inhibitors of DNA binding (*Id*) *1*, *2*, and *3* in a variety of cell types. Here, we examined mechanisms mediating BMP2-stimulated *Id3* transcription in murine gonadotropes. Using a combination of quantitative RT-PCR, promoter-reporter analyses, over-expression, and RNA interference approaches, we demonstrate that BMP2 signals via the BMPR2 and BMPR1A (ALK3) receptors and intracellular signaling proteins SMADs 1 and 5 to stimulate *Id3* transcription. We further define a novel 6-bp *cis*-element mediating BMP2- and SMAD-dependent transcription, though this site does not appear to bind SMADs directly. A specific DNA binding protein complex binds to this element, but its constituent protein(s) remain undetermined. Recently, a more distal enhancer was shown to mediate BMP4-induction of the human *ID3* gene in ovarian cancer cells. This enhancer is conserved in the murine gene and we demonstrate its role in BMP2-induced *Id3* promoter activity in gonadotropes. Conversely, the proximal *cis*-element defined here is also conserved in human *ID3* and we demonstrate its functional role in BMP2-induction of *ID3* transcription. Finally, we show that the two regulatory elements also mediate BMP2-induction of *Id3* promoter activity in murine fibroblasts. Collectively, we have defined a general mechanism whereby BMP2 regulates *Id3*/*ID3* transcription in different cell types and in different species.

1. Introduction

Bone morphogenetic proteins (BMPs) were originally identified as factors that induce ectopic bone and cartilage formation when implanted into muscular tissue (Urist, 1965). BMPs are now known to play diverse roles, for example, in osteoblast and chondrocyte differentiation, tooth development, kidney development, skin and hair development, myogenic differentiation, neural cell differentiation, and vascular homeostasis (Derynck and Miyazono, 2008). Over 20 BMP family members, all part of the larger transforming growth factor β (TGFβ) superfamily, have been identified and characterized (Derynck and Miyazono, 2008). Although BMPs exhibit highly conserved structures, they can be classified into several subgroups based on their structural homology. For example, BMP2 and BMP4 are highly similar and form one subgroup (Miyazono et al., 2010; Sieber et al., 2009). BMP2/4 signaling is initiated by binding to BMP type I serine/threonine receptors, ACVR1, BMPR1A and/or BMPR1B (Shimasaki et al., 2004). Type II receptors, such as BMPR2, are then recruited into the complex and phosphorylate the type I receptors (Allendorph et al., 2006; Miyazono, 1999). BMPs may also bind preassembled type I/type II receptor complexes (Gilboa et al., 2000; Nohe et al., 2002). BMP family members show some promiscuity in their receptor binding. For example, BMP2/4 preferentially signal through the type II receptor, BMPR2, but can use ACVR2 in its absence (Yu et al., 2005). The activated type I receptors phosphorylate intracellular signaling proteins, the most thoroughly characterized of which are the receptor-regulated SMADs (or R-SMADs), SMADs 1, 5, and 8. Once phosphorylated, R-SMADs form heteromeric complexes with the co-regulatory SMAD (SMAD4) and accumulate in the nucleus. SMADs then regulate target gene transcription by directly binding to DNA and interacting with different transcriptional co-activators or co-repressors (Massague et al., 2005; Miyazawa et al., 2002; Miyazono, 1999). The amino-terminal Mad homology 1 (MH1) domains of SMAD1/5/8 mediate their binding to GC-rich BMP response elements (BRE) in target genes (Ishida et al., 2000).

The inhibitors of DNA binding (*Id*) are well-characterized BMP response genes in a variety of cell types (Abe, 2006; Coppe et al., 2003; Derynck and Miyazono, 2008; Hua et al., 2006; Katagiri et al., 1994; Miyazono and Miyazawa, 2002; Peng et al., 2004; Samanta and Kessler, 2004; Vinals et al., 2004). Four *Id* sub-types

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* Corresponding author. Tel.: +1 514 398 2525; fax: +1 514 398 6705.
E-mail address: daniel.bernard@mcgill.ca (D.J. Bernard).

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(*Id1–4*), which exhibit similar, but not identical biological activities (Miyazono and Miyazawa, 2002), have been identified. *Id*s belong to the helix-loop-helix (HLH) family of transcriptional regulators. Unlike other HLH proteins, which can bind E-box *cis*-elements as homo- or hetero-dimers, *Id*s lack the basic amino acid domain necessary for DNA binding. *Id*s are instead conventionally thought to block transcriptional activity of bHLH proteins (such as Mash1, OLIG, NeruoD, and MyoD bHLH) by forming DNA-binding-deficient hetero-dimers (Hua et al., 2006; Katagiri et al., 1994; Samanta and Kessler, 2004; Vinals et al., 2004).

Id proteins have been implicated as effectors of BMP signaling in a variety of cellular systems and have a role in neurogenesis, angiogenesis, and bone formation (Abe, 2006; Coppe et al., 2003; Hua et al., 2006; Katagiri et al., 1994; Miyazono and Miyazawa, 2002; Peng et al., 2004; Samanta and Kessler, 2004; Vinals et al., 2004). Recently, we and others reported that BMPs are expressed in LβT2 cells, an immortalized murine gonadotrope cell line, and in adult murine pituitary. In the former, BMP2 can stimulate follicle-stimulating hormone β subunit (*Fshb*) gene expression alone and in synergy with activins (Huang et al., 2001; Lee et al., 2007; Otsuka and Shimasaki, 2002). BMP2 also induces *Id1*, *2*, and *3* mRNA expression in these cells and we demonstrated that BMP2 synergistically stimulates *Fshb* transcription with activins, at least in part, through the combined actions of *Ids 2* and/or *3* and SMAD3, a major effector of activin signaling (Bernard, 2004; Ho and Bernard, 2010). Here, we defined part of the mechanism whereby BMP2 regulates *Id3* transcription; ultimately providing a more complete understanding of BMP regulated *Fshb* expression.

2. Materials and methods

2.1. Reagents

Human recombinant (rh-) BMP2 and activin A were purchased from R&D Systems (Minneapolis, MN, USA). Gentamycin, 1× phosphate buffered saline (PBS), and Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate were purchased from Wisent (St-Bruno, Quebec, Canada). Random primers, MMLV-reverse transcriptase, RNasin, and deoxynucleotide triphosphates (dNTPs), and 1× Passive Lysis Buffer (PLB) were from Promega (Madison, WI, USA). Protease inhibitor tablets (CompleteMini), and Expand Long Template PCR System were purchased from Roche (Mississauga, ON, Canada). Aprotinin, leupeptin, pepstatin, phenylmethylsulphonylfluoride (PMSF), SB431542, EZview Red ANTI-HA M2 Affinity Gel (Cat. # E6779), mouse monoclonal HA (#H9658), mouse monoclonal β-actin (#A5441), rabbit monoclonal FLAG (#F3165) antibody, cycloheximide, actinomycin D, pancreatin, and collagenase were from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA) and enhanced chemiluminescence (ECL) Plus reagents were from GE Healthcare (Piscataway, NJ, USA). Lipofectamine/Plus, Lipofectamine 2000, Media 199 (M199), Hanks' Balanced Salt Solution (HBSS), TRIzol Reagent, and SYBRgreen Supermix for qPCR were from Invitrogen (Burlington, ON, Canada). Oligonucleotides were purchased from IDT (Coralville, IA, USA). ^{32}P-ATP was from Perkin Elmer (Boston, MA, USA). Short-interfering (si) RNAs were purchased from Dharmacon (Lafayette, CO, USA): Control (Cat. # D-001210-05), *Acvr1* (Cat. # D-042047-01); *Bmpr1a* (Cat. # D-040598-01); *Bmpr1b* (Cat. # D-051071-01); *Smad1* (Cat. # D-055762-01 and D-055762-02); *Smad5* (Cat. # D-057015-01); *Smad8* (Cat. # D-046344-01 and D-046344-02); *Acvr2* (Cat. # D-040676–01), *Acvr2b* (Cat. # D-040629–02), and *Bmpr2* (Cat. # D-040599–01). Formaldehyde (37%) was from Fisher Scientific (Ottawa, ON, Canada). ChampionChIP One-Day kit was purchased from SABiosciences [distributed by Cedarlane; Burlington, ON, Canada]. Anti-SMAD1 (Cat# sc-7965×) was from Santa Cruz (Santa Cruz, CA, USA) and normal mouse IgG (Cat. # 12-371) was from Millipore (distributed by Cedarlane).

2.2. Constructs

The expression constructs for rat ACVR1-HA, human FLAG-SMAD1, murine FLAG-SMAD4, and rat SMAD5 were provided by Dr. Teresa Woodruff (Northwestern University, Chicago, IL, USA). The latter was sub-cloned into a pcDNA3.0 vector bearing an N-terminal FLAG tag. Human BMPR1A-HA (Q233D) and mouse BMPR1B-HA (Q203D) were provided by Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan). Rat myc-SMAD8 and murine FLAG-SMAD5 were provided by Dr. C.H. Heldin (Ludwig Institute for Cancer Research, Sweden) and Dr. T. Watanabe (Tokyo University, Tokyo, Japan), respectively. The rat FLAG-SMAD8 construct was generated in-house by PCR using MYC-SMAD8 as template. The murine −1561/+15 *Id2*-luciferase and −886/+15 *Id3*-luciferase promoter-reporters were provided by Dr. Yoshifumi Yokota (University of Fukui, Fukui, Japan) (Karaya et al., 2005) and Dr. Robert W. Lim (University of Missouri-Columbia, DC, USA) (Yeh and Lim, 2000), respectively, and their 5'deletions generated by PCR as previously described (Bernard, 2004) (see Supplemental Table S1 for primers). The murine −3740/+24 *Id3*-luciferase reporter was generated from wild-type C57BL6/J mouse genomic DNA using the Expand Long Template PCR kit (Roche) and the primers indicated in Supplementary Table S1 and ligated into the *Mlu*I and *Xho*I sites in pGL3-Basic (Promega). The human *ID3* promoter-reporter constructs (−4104/+402, −1927/+402, −653/+402, and +36/+402) and parental pGL2-Basic vector were provided by Dr. Trevor Shepherd (University of Western Ontario, ON, Canada). Note that the numbering of the constructs has been modified here relative to that reported in Shepherd et al. (2008). These changes were made based on our sequencing of the ends of the constructs and comparing them to the human *ID3* mRNA and genomic sequences described in GenBank acc. #NM_002167 and NC_000001.10, respectively. Site-directed mutagenesis of the murine *Id3* and human *ID3* promoter-reporters as well as of the SMAD1 and SMAD8 siRNA-sensitive expression constructs was performed using the Stratagene QuikChange protocol (Agilent Technologies, Mississauga, ON, Canada). The GATA4-HA and dominant-negative GATA4 constructs were provided by Dr. Robert Viger (Université Laval, Québec City, Québec, Canada).

2.3. Cell culture and transfections

Immortalized murine gonadotrope LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego, CA, USA) and were cultured in 10% FBS/DMEM and 4 μg/ml gentamycin as described previously (Bernard, 2004). For luciferase assays, cells were plated in 24-well plates (2.5×10^5 cells per well) approximately 36 h prior to transfection. Cells were transfected with 450 ng reporter/well using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). Twenty-four hours after transfection, cells were washed in 1× PBS and then treated with ∼1 nM (25 ng/ml) BMP2 plus 10 μM SB431542 in DMEM or with 10 μM SB431542 in DMEM alone (control) for the indicated times. The ALK4/5/7 inhibitor SB431542 (Inman et al., 2002) was included to block any potential effects of endogenous activin B. However, it should be noted that inclusion of the inhibitor was not required to observe BMP2-induced *Id2* or *Id3* expression, and that any effects of activins are likely to be modest and mediated via mechanisms distinct from those underlying BMP2 responses (data not shown). In time-course experiments, the introduction of ligand was staggered so that protein lysates from different treatment groups were collected at the same time. In over-expression experiments, LβT2 cells cultured in 24-well plates were transfected with the reporter as described above plus 100 ng of each receptor or SMAD expression vector (or empty vector for controls) per well. Cells were changed to serum-free media and lysates collected 24 h later. In RNA interference (RNAi) experiments, siRNAs in 1× siRNA buffer (20 mM KCl, 6 mM HEPES-pH 7.5, and 0.2 mM $MgCl_2$) were transfected at a final concentration of 5 nM. Resulting data were normalized to cells transfected with the control siRNA. Twenty-four hours after transfection, cells were washed in 1× PBS and treated with BMP2 and SB431542 for 24 h as described above. Lysates were collected 24 h later to measure luciferase activity. LβT2 cells plated in 6-well plates (1×10^6 cells per well) approximately 48 h prior to treatment were washed in serum-free DMEM and then treated with 25 ng/ml BMP2 in DMEM with or without 5 μg/ml cycloheximide or 5 μg/ml actinomycin D for 1 h. Cells were washed with 1× PBS and total RNA was collected with TRIzol for qPCR analysis following the manufacturer's instructions. Nuclear extracts were collected from LβT2 cells cultured in 10-cm plates as previously described (Lamba et al., 2006) and used for gel shift experiments. For chromatin immunopreciptitation (ChIP) analyses (see details below), approximately 5×10^6 LβT2 cells cultured in 10-cm dishes for approximately 48 h were washed with 1× PBS and treated for 1 h with ∼2 nM (50 ng/ml) BMP2 plus 10 μM SB431542 in DMEM. Cells were fixed and harvested following the manufacturer's instructions (SABiosciences).

CHO and NIH3T3 cells were obtained from Dr. Patricia Morris (Population Council, New York, NY). CHO cells cultured in F-12/DMEM containing 10% FBS and 4 μg/ml gentamycin in 6-well plates were transfected when 70–80% confluent with 100 ng of the indicated FLAG-SMAD 1, 5, or 8 expression constructs and 10 nM siRNA using Lipofectamine/Plus for 6 h and then changed to growth media. Cell lysates were then harvested the following day for western blot analysis. CHO cells grown in 10-cm dishes were transfected using Lipofectamine/Plus and 4 μg of the indicated HA-tagged GATA4 and FLAG-tagged SMAD1 expression vectors. Protein lysates were harvested the following day for use in immunoprecipitation analyses (see below). NIH3T3 cells used for luciferase assays were cultured at 10^5 cells per well in 10% FBS/DMEM and 4 μg/ml gentamycin. Transfection protocols were identical to those used for the LβT2 cells.

2.4. Luciferase assays

Cells were washed with 1× PBS and lysed in 1× PLB. Luciferase assays were performed on an Orion II microplate luminometer (Berthold detection systems, Oak Ridge, TN, USA) using standard reagents (Bernard, 2004). All treatments were performed in duplicate or triplicate as described in the figure legends. Data are represented as means of means (+SEM or SD) from three or more independent experiments.

2.5. Reverse transcription and qPCR

Reverse transcription was performed on 1–2 μg of total RNA as previously described (Lee et al., 2007). qRT–PCR was performed on the resulting cDNA using the SYBRgreen Supermix following the manufacturer's instructions with a Corbett Rotorgene 6000 qPCR machine (Corbett Life Science). As results from replicate qPCR experiments were qualitatively similar, but sometimes quantitatively different (in terms of fold effects), we presented data from one representative experiment out of three individual experiments (Figs. 1 and 3(C)). The remaining two replicates in each experiment were presented as Supplementary Figs. S1 and S2. Expression of target genes was normalized relative to ribosomal protein L19 (*Rpl19*) in the same sample and presented relative to the no ligand control. Sequences of the qPCR primers for *Rpl19*, *Id2*, *Id3*, and *Bmpr1a* genes are shown in Supplementary Table S1.

2.6. Primary pituitary cultures and adenoviral infection

Male *Bmpr1a*$^{flox/flox}$ mice (Mishina et al., 2002) were sacrificed at 8 weeks of age in accordance with institutional and federal guidelines. Pituitaries were extracted, quartered using scalpel blades, and digested with collagenase for 2 h in a 36 °C shaking water bath. Cells were collected by centrifugation at 1000 × *g* for 5 min, resuspended in calcium free HBSS and further digested with 2× pancreatin for 15 min. Cells were collected by centrifugation, washed in complete M199 media four times, and then passed through a 40 μm filter cloth to remove cell debris. 10^5 cells were plated per well in 96-well dishes. Cells were cultured in 10% FBS/M199 medium for 36 h before infection with adenovirus expressing GFP or Cre-IRES-GFP (provided by Dr. Derek Boerboom, Université de Montréal, Canada). Infection was verified 24 h later using Zeiss Axio Observer A1 fluorescent inverted microscope to detect GFP expression. Cultures were then pre-treated with 10 μM SB431542 in 10% FBS/M199 for 24 h. Cells were then washed with 1× PBS and treated (in the absence of SB431542) with 25 ng/ml activin A or 50 ng/ml BMP2 in 2% FBS/M199 for 24 h. Total RNA was extracted with TRIzol (Invitrogen) following the manufacturer's instructions for qPCR analysis. Genomic DNA was also prepared from the same extracts and subjected to genotyping analysis by PCR (to confirm recombination) using primer sets Fx1/4 or Fx2/4 shown in Supplemental Table S1 with an annealing temperature of 55 °C for 35 cycles.

2.7. Immunoprecipitation

CHO cells were washed with 1× PBS and whole cell protein extracts (WCE) prepared with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and centrifuged at 10,000 rpm for 15 min at 4 °C to remove insoluble material. WCEs were subjected to immunoprecipitation using EZview Red ANTI-HA M2 Affinity Gel following the manufacturer's instructions. In brief, WCEs were incubated with the affinity gel on a rotating platform overnight at 4 °C to allow binding of HA-GATA4 to the ANTI-HA affinity gel. The affinity gel was then incubated in 1× HA peptide (Cat. # I2149) solution on a rotating platform for 45 min at 4 °C to elute gel-bound proteins. The eluted proteins were then analyzed by western blot using anti-FLAG and anti-HA (see below).

2.8. Western blotting

Cells were washed with 1× PBS and WCE prepared with 1× RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 6.8, 2 mM EDTA, 50 mM sodium fluoride, and CompleteMini Protease Inhibitor Cocktail Tablets) and centrifuged at 13,000 × *g* for 0.5 h at 4 °C to remove insoluble material. WCEs were subjected to western blot analyses as previously described (Bernard, 2004). Briefly, equivalent amounts of protein were separated by SDS-PAGE and transferred to Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Blots were probed with the indicated antibodies using standard techniques (Bernard, 2004; Bernard et al., 2006).

2.9. Electrophoretic mobility shift assays

Gel shift experiments were performed as described (Therrien and Drouin, 1993), with minor alterations to the protocol. Briefly, nuclear protein concentrations were determined by Bradford assay (BioRad). Nuclear proteins (3–5 μg) were incubated for 20 min at room temperature with 50 fmol of ^{32}P-ATP end-labeled double-stranded DNA probes corresponding to the indicated fragments (see Fig. 5(B) and (C), S6A) of the murine *Id3* promoter in binding buffer (25 mM HEPES pH 7.2, 150 mM KCl, 5 mM dithiothreitol, 10% glycerol). Five hundred ng salmon sperm DNA or 0.5–1 μg poly(dI)·poly(dC) was used in the binding buffer as non-specific competitor. In competition experiments, reactions were assembled at room temperature and incubated for 10 min with 100-fold molar excess unlabeled (cold) competitor probes prior to the addition of the radio-labeled probe. Reactions were then run on 5% polyacrylamide gels (44:0.8 acrylamide:bis-acrylamide) in 40 mM Tris–HCl/195 mM glycine (pH 8.5) at 200 V for 3–5 h at 4 °C. Gels were dried and exposed to X-ray film (Kodak).

2.10. Chromatin immunoprecipitation (ChIP) assays

Approximately 4–6 × 10^6 LβT2 cells per 10-cm culture dish were harvested for each experiment according to the manufacturer's guidelines (SABiosciences). In brief, crosslinking was performed for 10 min at room temperature with 1% formaldehyde diluted in 1× PBS. Cells were then quenched in 1× glycine for 5 min, washed twice with cold 1× PBS, and harvested with 1× PBS containing protease inhibitor cocktail using a rubber policeman. Cells were then collected by centrifugation and lysed with the provided lysis buffer. Cell contents (DNA/protein complexes) were sonicated using a Misonix Sonicator 3000 (Mandel, Guelph, ON, Canada) to obtain chromatin fragments around 750 bp (35 s; 5 s ON, 10 s OFF). Lysates were collected by centrifugation at high speed for 10 min at 4 °C. Two-hundred microliter aliquots of sheared chromatin were subjected to pre-clearing using Protein A beads for 1 h at 4 °C. Ten microliter aliquots of each sample were removed to be used as input control. The remaining chromatin fractions were subjected to immunoprecipitation using 4 μg of SMAD1 Ab or 4 μg of the control normal mouse IgG overnight at 4 °C with rotation followed by 1 h incubation with Protein A beads. Beads were washed with the provided wash buffers (1–4) in five sequential steps at room temperature. Reverse crosslinking was performed in a 45 °C shaking water bath with 20 μg proteinase K diluted in the provided elution buffer for 30 min, followed by a 10 min incubation at 95 °C. DNA purification was performed using the provided DNA spin columns and eluted with elution buffer. Quantitative real-time PCR was performed using 4–6 μl of the eluted DNA for 40 cycles using the SYBRgreen Supermix following the manufacturer's instructions with a Corbett Rotorgene 6000. Data were analyzed following SABiosciences ChIP quantitative PCR analysis guidelines by normalizing against the input and samples immunoprecipitated with control IgG (equivalent to the ΔΔCt method). The resulting data represent the mean of three independent experiments ($N = 1$ per experiment). Sequences of the qPCR primers for the various *Id3* promoter fragments are shown in Supplemental Table S1.

2.11. Data analyses

In all cases, reporter assay data from three replicate experiments were highly similar. Therefore, means of treatment replicates within each experiment were calculated to generate a single value per treatment per experiment. The data from replicate (three or more) experiments were then used for statistical analyses. Figures shown for qPCR data are representative graphs, unless otherwise indicated; experiments were performed three times with results showing similar trends. Data are presented as fold-change from the control condition (no ligand and/or transfection with empty vector alone). Differences between means were compared using one-, two-, or three-way analyses of variance followed by post hoc pair-wise comparison with Bonferroni adjustment where appropriate (Systat 10.2, Richmond, CA, USA). Bars or values with different symbols were statistically different, whereas those sharing symbols did not differ. Significance was assessed relative to $p < 0.05$.

3. Results

3.1. BMP2 stimulates Id2 and Id3 transcription in gonadotropes

We previously reported that BMP2 stimulates increases in *Id1*, *Id2*, and *Id3* mRNA levels in LβT2 cells (Ho and Bernard, 2010). Here, we established that the increases in *Id2* and *Id3* mRNA levels reflect direct transcriptional responses. BMP2-stimulated *Id2* and *Id3* mRNA expression was completely blocked by pre-treatment with the transcriptional inhibitor actinomycin D (Fig. 1 and Supplementary Fig. S1). In contrast, pre-treatment with the translational inhibitor cycloheximide did not block BMP2-stimulated *Id2* or *Id3* expression. These data suggest that *Id2* and *Id3* are BMP2 immediate-early response genes in LβT2 cells.

3.2. The murine Id3 promoter is time- and dose-dependently stimulated by BMP2

To uncover mechanisms mediating BMP2-induced *Id2* and *Id3* transcription, we turned to promoter-reporter assays. When transfected into LβT2 cells, the murine −1561/+15 *Id2*-luciferase reporter (Karaya et al., 2005) was unresponsive to BMP2 (data not shown). It is possible that critical regulatory sequence was not present within the promoter fragments used. In contrast, a murine −886/+15 *Id3*-luciferase reporter (Yeh and Lim, 2000) was time (Fig. 2(A)) and concentration-dependently stimulated by BMP2 (Fig. 2(B)). Induction of reporter activity (Fig. 2(A)) showed slower kinetics than did induction of the endogenous gene (Fig. 1); how-

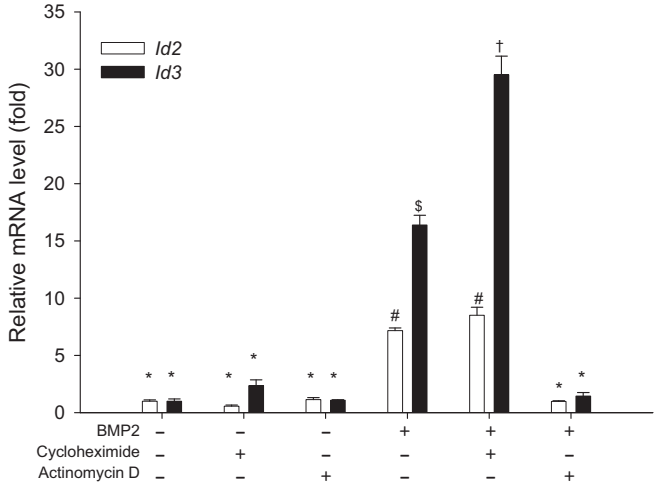

Fig. 1. LβT2 cells seeded in 6-well plates were pre-treated with 5 μg/ml cycloheximide or actinomycin D for 15 min prior to 25 ng/ml BMP2 treatment for 1 h. Changes in *Id2* and *Id3* mRNA expression were measured by qRT-PCR. Data are normalized to the housekeeping gene, *Rpl19*, and presented as fold change in mRNA expression relative to untreated cells. The figure shown is representative of three replicate experiments (see Supplemental Fig. S1 for the other replicates). *Id2* and *Id3* mRNA transcripts were analyzed separately. Here and in subsequent figures, bars with different symbols were statistically different, whereas those sharing symbols did not differ.

ever, differences in both the nature and sensitivity of the assays might explain (at least in part) this apparent discrepancy. The empty vector, pGL3-Basic, was not regulated by BMP2. It should be noted that the promoter itself, in the absence of BMP2, conferred significant basal reporter activity (compare the open and closed symbols at time 0 or in the absence of BMP2 in Figs. 2(A) and (B)). Based on these observations, subsequent reporter assays were limited to the *Id3* promoter and treatments were performed with 25 ng/ml BMP2 for 24 h. Next, 5′ deletions were used to define the minimally responsive reporter. Truncations from −886 to −568 did not significantly modify fold BMP2 induction (Fig. 2(C) and data not shown). However, further deletion to −502 completely abrogated the BMP2 response, while maintaining basal reporter activity. The minimal promoter-reporter, −568/+15 *Id3*-luc, was used in subsequent experiments.

3.3. BMP2 signals preferentially through BMPR1A and BMPR2 to regulate Id3 promoter activity

To determine the relevant signaling receptor(s) in this system, we used siRNAs to knock down endogenous expression of each of the type I receptors for BMP2 in LβT2 cells, and measured the fold BMP2 stimulation of the minimal *Id3* promoter-reporter. All the relevant BMP type I receptors are expressed in these cells (Ho and Bernard, 2010; Lee et al., 2007; Otsuka and Shimasaki, 2002). The *Bmpr1a* siRNA abolished BMP2-stimulated *Id3* promoter activity, whereas the *Acvr1* and *Bmpr1b* siRNAs were without effect (Fig. 3(A)). The sequence specificity and efficiency of all of the siRNAs were previously validated (Ho and Bernard, 2009). These data suggests that BMP2 preferentially signals through the type I receptor, BMPR1A, to stimulate *Id3* expression in LβT2 cells. In complementary assays, the *Id3* promoter was transfected together with different constitutively active type I receptors (Gln→Asp) (Hoodless et al., 1996). *Id3* promoter activity was stimulated significantly by BMPR1A-QD, less so with BMPR1B-QD, and not at all by ACVR1-QD (Fig. 3(B)). This is consistent with the idea that BMPR1A-mediated signaling induces *Id3* transcription.

To determine whether BMPR1A is important for BMP2-induced *Id3* expression in a more physiological context, we prepared primary pituitary cultures from floxed *Bmpr1a* mice (Mishina et al., 2002). Cells were infected with adenoviruses expressing GFP (control) or Cre recombinase and GFP (from a bi-cistronic mRNA). The cultures were then treated with activin A or BMP2 for 24 h. In control cells, BMP2 (but not activin A) stimulated an increase in *Id3* mRNA expression (Fig. 3(C) and Fig. S2), consistent with our results from LβT2 cells (Fig. 1, (Ho and Bernard, 2010), and data not shown). In cells transduced with Cre expressing virus, the BMP2 effect was abrogated (Fig. 3(C) and Fig. S2). Analysis of genomic DNA and RNA confirmed recombination of the *Bmpr1a* gene and the associated depletion of *Bmpr1a* mRNA in these cells (Fig. S3). These data suggest that BMP2 signals through BMPR1A to stimulate *Id3* transcription in murine pituitary cells.

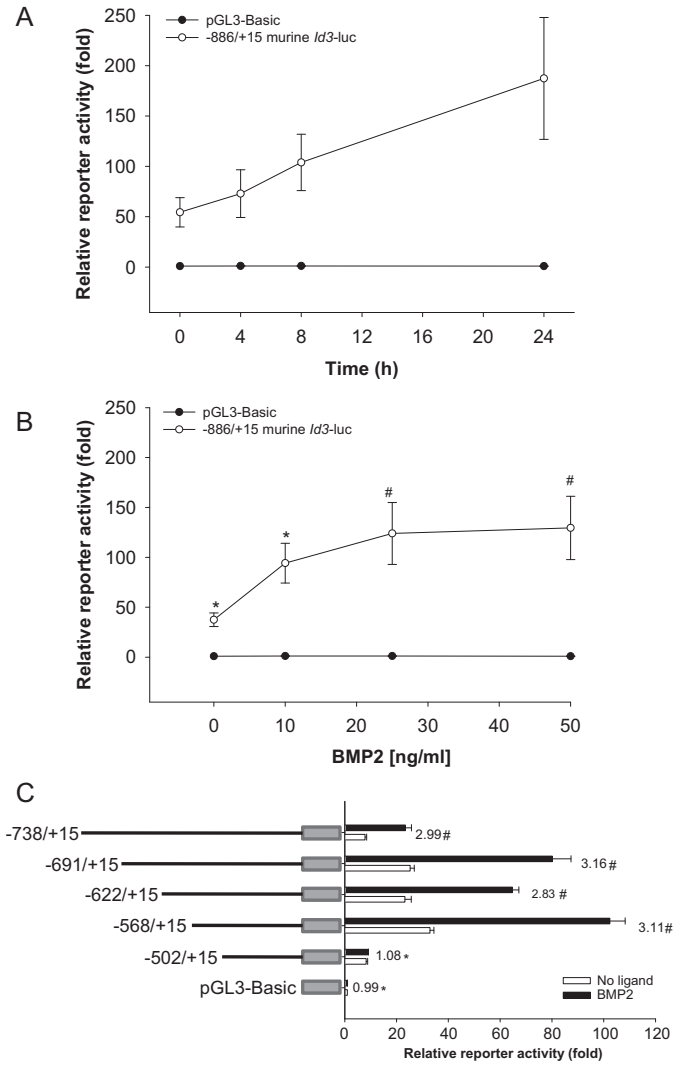

Fig. 2. (A) LβT2 cells seeded in 24-well plates were transfected with murine −886/+15 *Id3*-luc and treated with 25 ng/ml BMP2 for 0, 4, 8, or 24 h in serum-free medium containing 10 μM SB431542. (B) LβT2 cells seeded and transfected as above were treated with 0, 10, 25, or 50 ng/ml of BMP2 for 24 h in serum-free medium containing 10 μM SB431542. In both panels, treatments were performed in triplicate, the data reflect the mean (+SEM) luciferase activity of three independent experiments and are presented relative to the control group, in which the cells were transfected with the pGL3-Basic vector and no ligand was applied. (C) LβT2 cells seeded in 24-well plates were transfected with the indicated 5′ deletions of the murine *Id3* promoter-reporter and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to pGL3-Basic transfected cells in the absence of ligand. Values beside the bars represent the fold stimulation by BMP2 for each reporter.

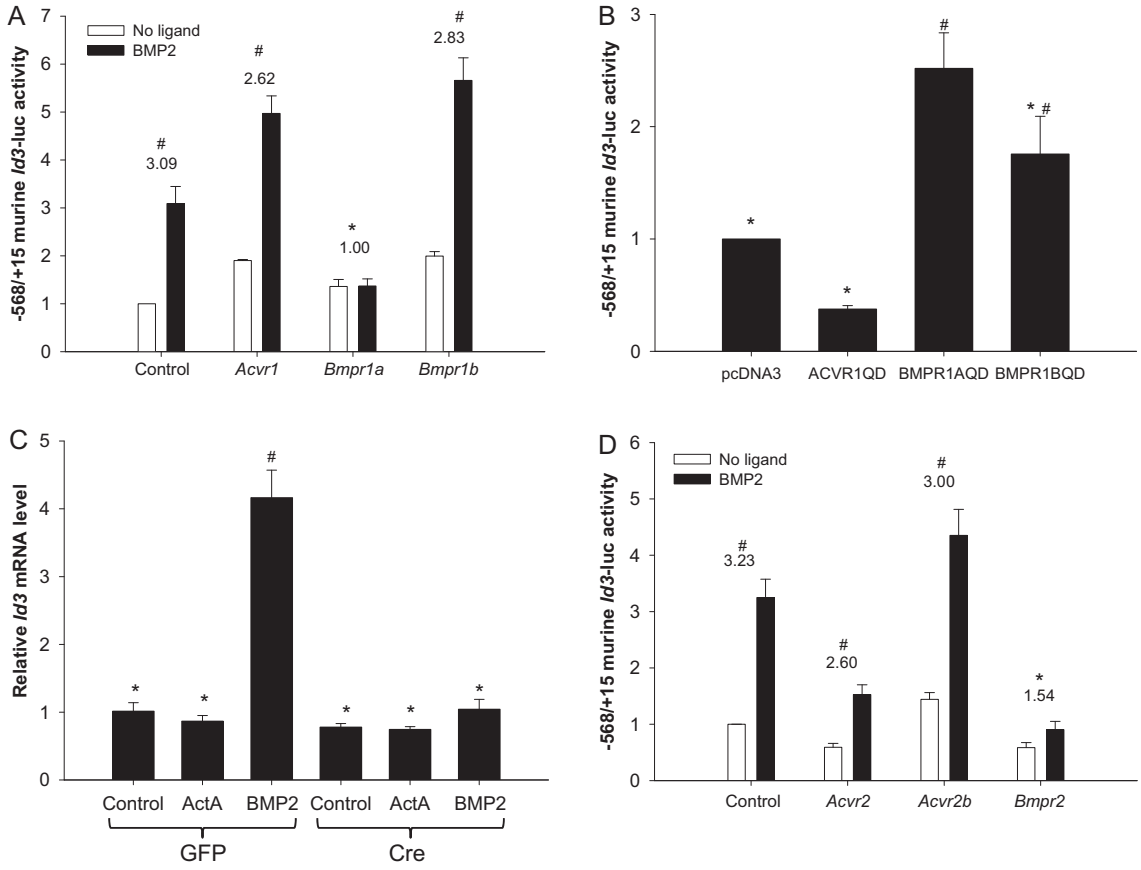

Fig. 3. (A) LβT2 cells seeded in 24-well plates were transfected with murine −568/+15 *Id3*-luc and 5 nM of the control short interfering RNA (siRNA) or siRNAs for the indicated BMP type I receptors. Cells were then treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which control siRNA was transfected but no ligand was added. Values above the bars represent the fold stimulation by BMP2. (B) LβT2 cells seeded in 24-well plates were transfected with 100 ng/well of the indicated constitutively active type I receptor constructs and murine −568/+15 *Id3*-luc. Cells were starved in serum-free medium for 24 h prior to analysis. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which the empty vector was transfected. (C) Pituitary cultures from transgenic *Bmpr1a*$^{flox/flox}$ mice infected with adenovirus expressing GFP or Cre recombinase and GFP were pre-treated with 10 μM SB431542 and then treated with 25 ng/ml activin A or 50 ng/ml BMP2. Changes in *Id3* mRNA expression were measured by qRT-PCR. Data are presented relative to untreated cultures infected with GFP-expressing virus. Treatments were performed in triplicate. The figure shown is from a representative experiment. Results of the replicate experiments are presented in Supplemental Fig. S2. (D) LβT2 cells seeded in 24-well plates were transfected with murine −568/+15 *Id3*-luc and 5 nM of the control siRNA or siRNAs for the indicated BMP type II receptors and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no siRNAs or ligands were included. Values above the bars represent the fold stimulation by BMP2.

Having established BMPR1A as the relevant type I receptor, we next sought to determine with which type II receptor it cooperates to mediate BMP2 activity. Knock down of *Acvr2* or *Acvr2b* with specific siRNAs did not affect BMP2-induced *Id3* transcription, whereas depletion of *Bmpr2* significantly diminished the BMP2 response (Fig. 3(D)). Collectively, these data suggest that BMP2 preferentially signals through BMPR1A and BMPR2 to stimulate *Id3* expression in gonadotropes.

3.4. BMP2 signals through SMAD1/5 to regulate Id3 promoter activity

To determine the relevant signal transducers in this system, we used siRNAs to knockdown expression of each of the BMP R-SMADs. Depletion of endogenous SMAD1 or SMAD5 significantly reduced BMP2-stimulated *Id3* promoter-reporter activity, whereas SMAD8 knockdown had little or no effect (Fig. 4(A)). To confirm the efficacy of the siRNAs, we examined the effects of the siRNAs on SMAD1/5/8 protein expression. Under our assay conditions, transfection efficiency of LβT2 cells is insufficient to obtain an accurate measure of the extent of RNAi-mediated knockdown of mRNA/protein expression on a per cell basis (Bernard, 2004). Therefore, to validate the *Smad5* siRNA, we over-expressed a murine FLAG-SMAD5 construct, which is sensitive to the siRNA, and a rat FLAG-SMAD5 construct, which is resistant to the siRNA, in CHO cells in the presence or absence of the *Smad5* siRNA (Fig. S4). We did not have a murine SMAD1 expression vector to validate the *Smad1* siRNA. Therefore, we used an available human-SMAD1 construct and modified its nucleotide sequence to make it sensitive to the murine *Smad1* siRNA. Two different targeting siRNAs were used; therefore, two siRNA resistant constructs were generated. This was similarly done with an available rat SMAD8 construct. The siRNA-resistant and -sensitive constructs were expressed in CHO cells in the presence or absence of their respective *Smad* siRNAs. Western blot analysis confirmed that each siRNA potently inhibited protein expression of its siRNA-sensitive target, but not the siRNA-resistant form of the construct. BMP2-induced *Id3* reporter activity, which was inhibited by the *Smad5* siRNA, was rescued by over-expression of an siRNA-resistant rat SMAD5 (Fig. S5). In complementary assays, the *Id3* promoter-reporter was transfected together with different R-SMADs alone or in combination with SMAD4, and then treated with BMP2 (Fig. 4(B)). BMP2 induction of *Id3* promoter activity was potentiated most robustly when SMAD4 was co-expressed with SMAD1 or SMAD5. Together, these data suggest that BMP2

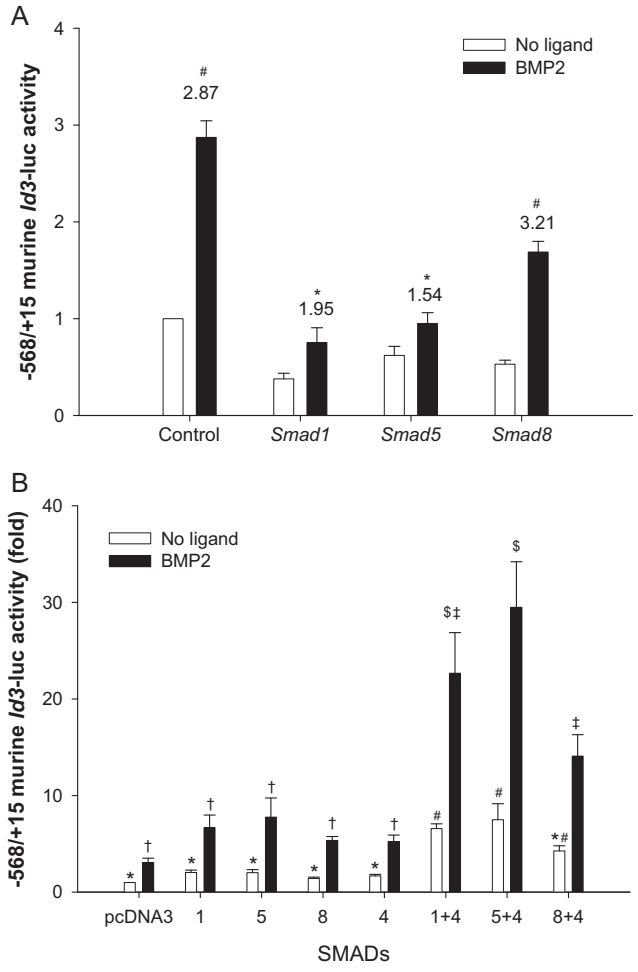

Fig. 4. (A) LβT2 cells seeded in 24-well plates were transfected with murine −568/+15 *Id3*-luc and 5 nM of the indicated siRNAs and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group, in which no siRNAs or ligand were included. Values above the bars represent the fold stimulation by BMP2. (B) LβT2 cells seeded in 24-well plates were transfected with 100 ng/well of the indicated SMAD expression constructs and murine −568/+15 *Id3*-luc. Cells were treated with 25 ng/ml BMP2 in serum-free medium for 24 h. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to the control group (pcDNA3, no ligand). BMP2 treated and untreated groups were analyzed separately.

signals through complexes of SMAD1/4 and/or SMAD5/4 to regulate murine *Id3* transcription in gonadotrope cells.

3.5. Identification of a novel BMP2 response element in the murine Id3 promoter

Above, we defined a BMP2 responsive region of the murine *Id3* promoter in the interval between −568 and −502 relative to the start of transcription (Fig. 2(C)). Using additional 5′ deletions, we further defined two BMP2 responsive regions; one between −568/−548 and the other between −528/−502 (Fig. 5(A)). Several nuclear proteins from LβT2 cells could bind within these intervals as revealed by electrophoretic mobility shift assays (EMSAs) (Fig. 5(B), see lanes 2 and 12). Specificity of complex binding was demonstrated by co-incubation with unlabeled competitor probes (compare lanes 2 and 3; and lanes 12 and 15). Complex formation was unaffected by BMP2 treatment (data not shown).

To identify the specific base pairs (bp) mediating protein complex binding, 3-bp scanning mutations were first introduced into −528/−502 competitor probes (Fig. 5(C)). All four complexes (labeled A–D at left) were competed by the wild-type probe (lane 13). In contrast, probes bearing mutations 4 and 5 (lanes 6 and 7) failed to compete for complex D, suggesting that bp −519/−514 mediate binding of the protein(s) in this complex. Results with the other competitors suggest that the protein(s) in complex C bind to bp −525/−514 (lanes 4–7), in complex B to bp −525/−517 (lanes 4–6), and in complex A to bp −516/−511 (lanes 7 and 8). Mut2 through Mut6 were next individually introduced into the murine −568/+15 *Id3* promoter-reporter. None of the mutations affected basal reporter activity in LβT2 cells. In contrast, Mut4 and Mut5 both impaired BMP2-stimulated promoter activity (Fig. 5(D)). These data suggest that binding of the protein(s) in complex D (though not BMP2-regulated in these assays) may be required for BMP2-induced promoter activity. The same approach was used to identify the base pairs required for protein complex formation within the −568/−542 interval; however, the competition data were less clear (Fig. S6A) and introduction of the corresponding mutations into the *Id3* promoter did not significantly impair reporter activity (Fig. S6B).

Supershift experiments in EMSA (data not shown), DNA affinity pull-down (DNAP; data not shown), and chromatin immunoprecipitation analyses (ChIP; Fig. S7A) all failed to demonstrate SMAD1/5 binding to this element. Nonetheless, both Mut4 (Fig. 5(E)) and Mut5 (data not shown) inhibited SMAD5/4 induction of promoter activity. Based on *in silico* analyses, the base pairs mediating complex D binding resembled a GATA factor binding site. Furthermore, both super-shift and DNAP analyses suggested that one or more GATA proteins could bind this element (data not shown). However, transfection of a dominant-negative GATA4 had no impact on basal or BMP2-regulated promoter activity (data not shown) and we were unable to demonstrate an interaction between SMAD1 or 5 and GATA4, despite replicating previous work showing that SMAD3 and GATA4 interact (Belaguli et al., 2007) (Fig. S7B and data not shown). Although our data suggest that GATA proteins may be part of complex D, functional data did not corroborate a role for them, so the identity of the components of complex D mediating BMP responsiveness remains unresolved at present.

3.6. Conservation of the BMP2 response element in the human ID3 promoter

In the course of our investigations, a mechanism for BMP4-stimulated human *ID3* expression in ovarian cancer cells (CaOV3) was described (Shepherd et al., 2008). In that report, an enhancer was described upstream of the promoter region we investigated here in mouse. We obtained the reporter constructs used in Shepherd et al. (2008) and examined their BMP2 induction in LβT2 cells (Fig. 6(A)). The full-length human reporter (−4104/+402) was robustly stimulated by BMP2 and the fold-induction (though not basal activity) was diminished by truncation to −1927, which removes the distal enhancer. However, the −1927 and −653 reporters were still induced by BMP2 and the BMP2 response was lost only following further truncation to +36. The 6-bp (−519/−514) mediating BMP2 induction of the murine *Id3* promoter (defined above) are perfectly conserved in the human gene (−188/−183) (Fig. S8). To determine if these base pairs also play a role in BMP2 induction of the human *ID3* promoter, we introduced mutations comparable to Mut4 and Mut5 in the murine *Id3* promoter into the minimal *hID3* promoter-reporter construct (−653/+402 *ID3*-luc) (Fig. 6(B)). Similar to what we observed with the murine *Id3* promoter, both mutations significantly impaired BMP2 induction of human *ID3* promoter activity.

3.7. The BRE in the human distal enhancer is conserved in the murine Id3 promoter

In silico analyses suggested that the upstream enhancer in the human *ID3* gene might be conserved in murine *Id3* (Fig. S8). As

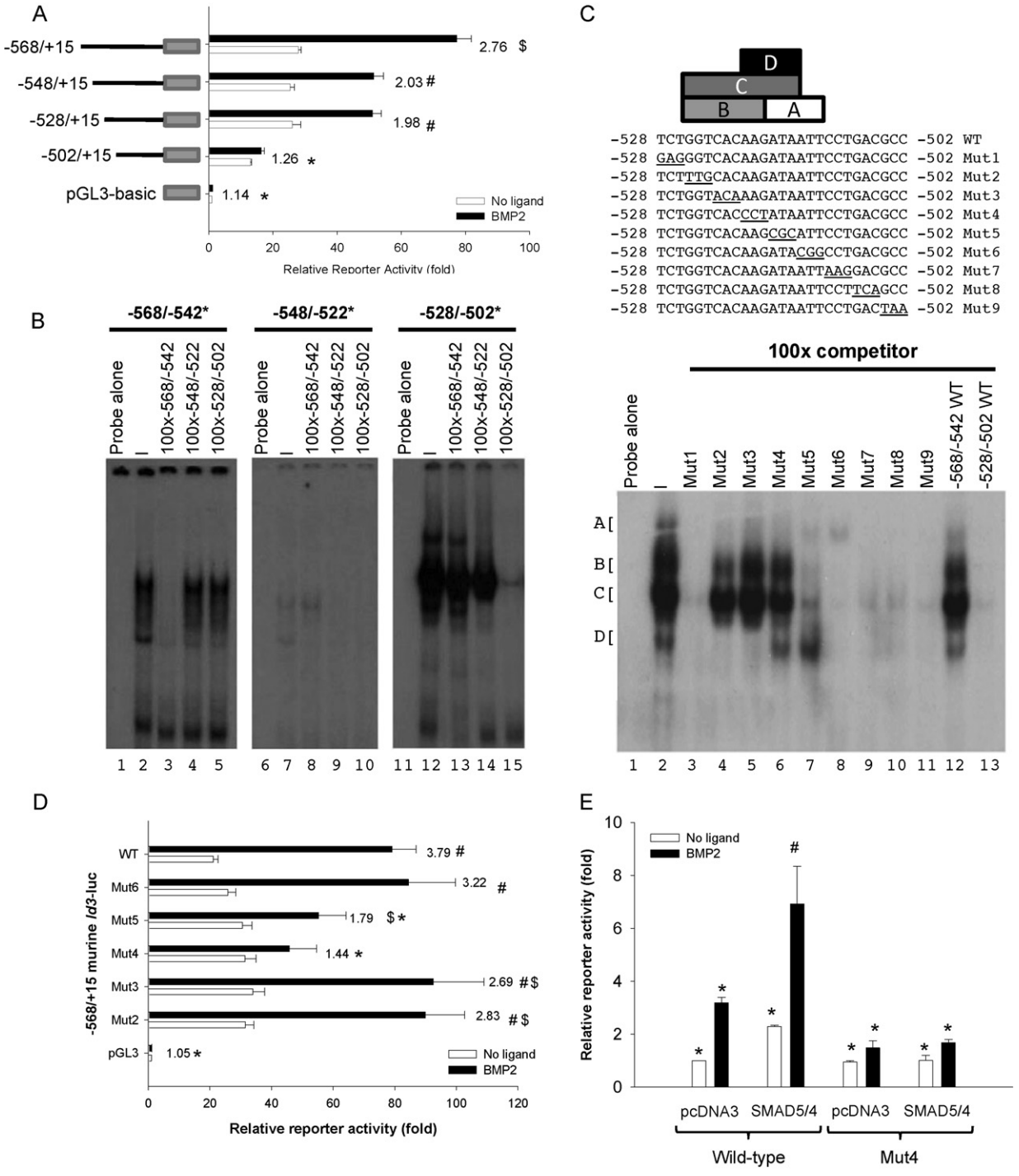

Fig. 5. (A) LβT2 cells seeded in 24-well plates were transfected with different lengths of murine *Id3* promoter-reporters and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with the empty vector, pGL3-Basic. Values beside the bars represent the fold stimulation by BMP2 for each reporter. (B) EMSAs were performed with the indicated radio-labeled (*) probes corresponding to −568/−502 of the murine *Id3* promoter. Nuclear extracts were prepared from LβT2 cells treated with 10 μM SB431542 in serum-free medium. In competition experiments, unlabeled probes were included at 100× higher concentration than the radio-labeled probes. The figure shown is representative of three experiments. Free probes are not pictured. (C) EMSAs were performed as in panel B with the −528/−502 radio-labeled probe. Here, competitions were performed with 3-bp scanning mutants of the −528/−502 probe (Mut1–9, shown at top). The schematic at the top reflects the relative positions of the nucleotides mediating binding of complexes A through D. (D) Mut2 through Mut6 defined in panel C were introduced into the −568/+15 *Id3* reporter and the constructs transfected into LβT2 cells seeded in 24-well plates. Cells were treated with BMP2 as described in panel A. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL3-Basic. Values beside the bars represent the fold stimulation by BMP2 for each reporter. (E) LβT2 cells seeded in 24-well plates were transfected with wild-type or Mut4 −568/+15 *Id3*-luc and 100 ng/well of each SMAD5 and SMAD4. Cells were then treated with 25 ng/ml BMP2 in serum-free medium containing 10 μM SB431542 for 24 h. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with the wild-type reporter and pcDNA3.

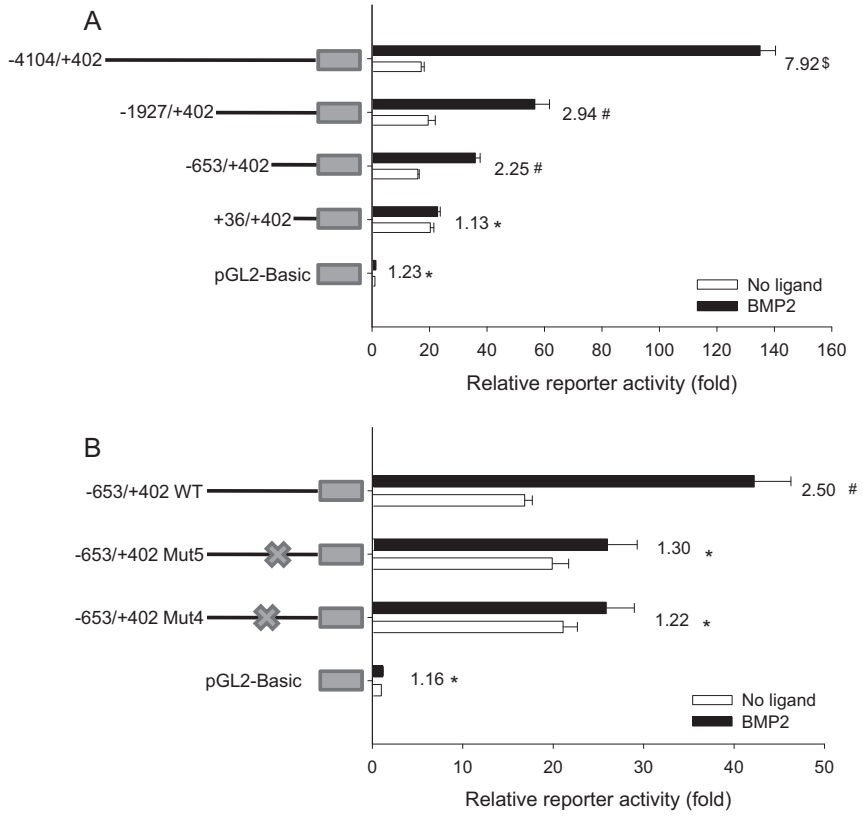

Fig. 6. (A) LβT2 cells seeded in 24-well plates were transfected with different lengths of human *ID3* promoter-reporters and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with the empty vector, pGL2-Basic. (B) The equivalents of Mut4 and Mut5 (each represented as an X on the promoter) in the murine *Id3* promoter were introduced into the human −653/+402 *ID3* promoter-reporter and the constructs transfected into LβT2 cells seeded in 24-well plates. Cells were then treated as in panel A. Treatments were performed in triplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL2-Basic.

the relevant sequence maps approximately 3.6 kb upstream of the transcriptional start site in the murine *Id3* gene, it was not represented in the reporter constructs used in our initial analyses. We therefore generated a larger murine *Id3* promoter-reporter and observed that it was more strongly induced by BMP2 in LβT2 cells than was the −568/+15 reporter used above (Fig. 7(A)). The analysis of the human gene revealed a BMP response element (BRE) within a distal enhancer (−2632/−2625) that when mutated (TGGCGCC→TGGTGCT) greatly reduced the fold BMP4 response (Shepherd et al., 2008). We identified the same sequence in the murine gene (−3283/−3276) (Fig. S8) and observed through ChIP analysis that BMP2 stimulated recruitment of SMAD1 to this region of the endogenous *Id3* gene in LβT2 cells (Fig. S7A). Mutation of the distal BRE in the longer murine *Id3* reporter greatly reduced, but did not abolish, BMP2 induction (Fig. 7(B)). Introduction of Mut4 or Mut5 in the context of the longer reporter also reduced the BMP2 response, though to a lesser extent than the mutation to the distal BRE (Fig. 7(B)). Importantly, the BRE and Mut4 mutations in combination almost completely blocked BMP2-stimulated reporter activity, demonstrating that both the proximal and distal BREs work in concert to mediate the BMP2 response.

3.8. The mechanism of BMP2-regulated Id3 transcription is conserved in fibroblasts

The above analyses were conducted exclusively in LβT2 or primary pituitary cells. To determine whether or not the described mechanisms were cell-type specific, we examined BMP2-stimulated promoter activity in NIH3T3 cells, the first cell type in which BMPs were shown to stimulate *Id3* expression (Barone et al., 1994; Christy et al., 1991). Both the 0.57 and 3.7 kb murine *Id3* reporters were induced by BMP2 in NIH3T3 cells (Fig. 7(C)). As in LβT2 cells, mutations made simultaneously to the distal and the proximal BREs abrogated BMP2 induction (Fig. 7(D)), demonstrating conservation of the mechanism.

4. Discussion

In the current study, we determined that BMP2 signals preferentially through the type I receptor, BMPR1A, and type II receptor, BMPR2, to regulate *Id3* transcription in LβT2 cells. BMPR1A also mediates BMP2-induced *Id3* expression in primary pituitary cultures. Further, we showed that the BMP response in LβT2 cells is mediated through SMAD1/5 and requires a conserved promoter element (−519/−514 in mouse; −188/−183 in human). Finally, we found that *Id3* transcription is further enhanced by a distal BRE first described in the human *ID3* gene (Shepherd et al., 2008).

*Id*s are well-known BMP responsive genes. A mechanism for BMP-stimulated *Id1/ID1* expression has been described. Selective deletion of *Bmpr1a* in murine endocardium suggests that BMPs preferentially signal through BMPR1A to stimulate *Id1* expression (Kaneko et al., 2008). Depletion of *Bmpr2* in cultured cells by siRNA or via Cre-mediated recombination in mice suggests that BMPR2 plays a crucial role in the sustained induction of *Id1* expression by BMP4 in vascular smooth muscle cells (Yang et al., 2008; Yu et al., 2008). The data reported here similarly define BMPR1A and

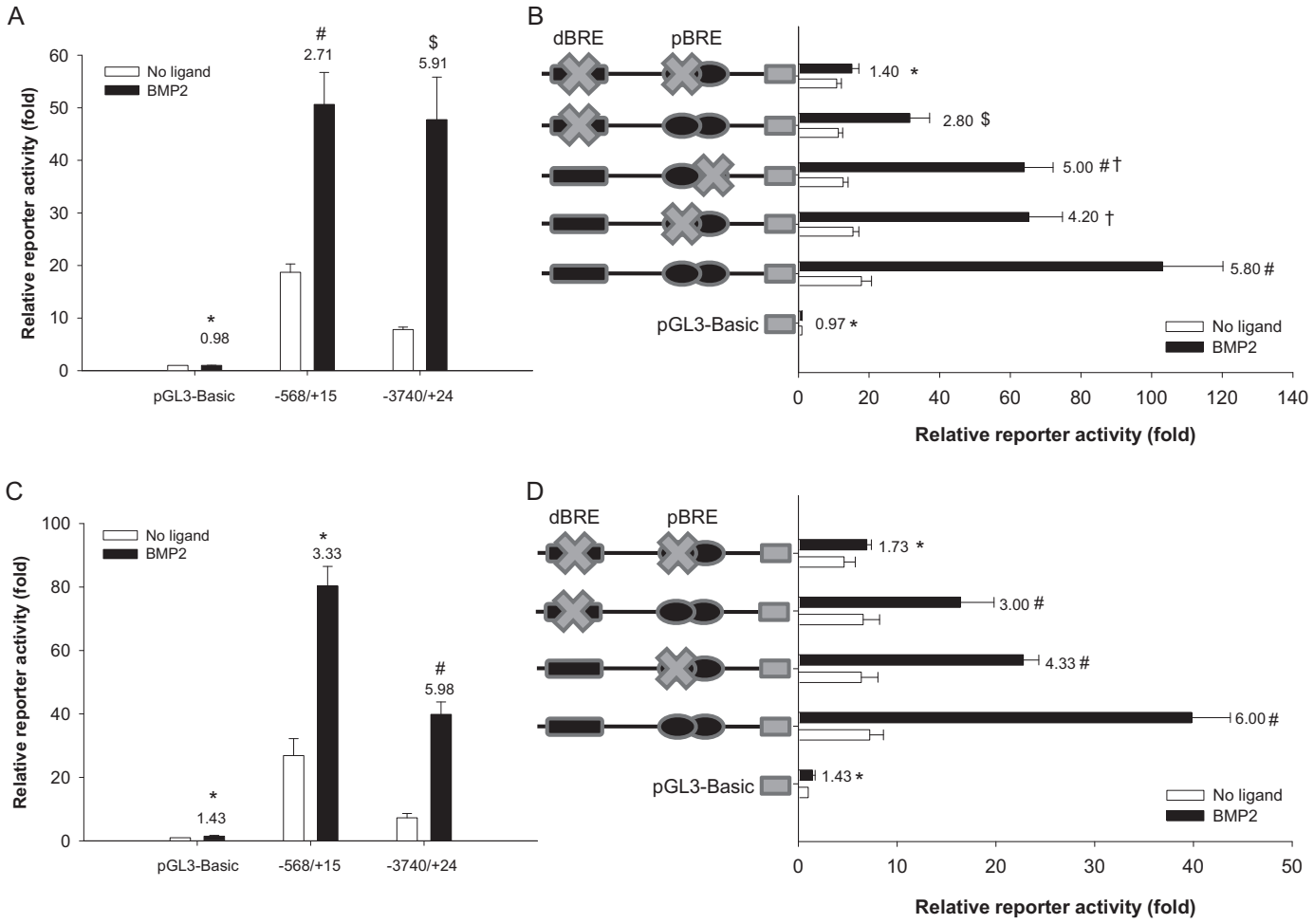

Fig. 7. (A) LβT2 cells seeded in 24-well plates were transfected with varying lengths of murine *Id3* promoter-reporters and treated with 25 ng/ml BMP2 for 24 h in serum-free medium containing 10 μM SB431542. Treatments were performed in triplicate. The data reflect the mean (+SEM) of nine independent experiments and are presented relative to the untreated cells transfected with the empty vector, pGL3-Basic. (B) Mutations were introduced to the distal or proximal BRE in the murine −3740/+24 *Id3*-luc alone or together, and the constructs transfected into LβT2 cells seeded in 24-well plates. The X in the dBRE represents the mutation TGGCGCC→TGGTGCT, whereas the X in pBRE represents Mut4 (left X) or Mut5 (right X) as described in Fig. 5(C). Cells were then treated as in panel A. Treatments were performed in duplicate. The data reflect the mean (+SEM) of three independent experiments and are presented relative to untreated cells transfected with pGL3-Basic. (C) NIH3T3 cells seeded in 24-well plates were transfected and treated as in panel A. Treatments were performed in triplicate. The data reflect the mean (+SEM) of four independent experiments and are presented relative to untreated cells transfected with pGL3-Basic. (D) NIH3T3 cells seeded in 24-well plates were transfected and treated as in panel B. Treatments were performed in duplicate. The data reflect the mean (+SEM) of four independent experiments and are presented relative to untreated cells transfected with pGL3-Basic.

BMPR2 as transducers of the BMP2 signal to the *Id3* promoter in gonadotrope cells. *Id3* mRNA expression is abolished in the endocardium of conditional *Bmpr1a* knockout mice (Kaneko et al., 2008), suggesting that BMPR1A is likely necessary for *Id3* expression in a variety of cell types. The type I receptors ACVR1 and BMPR1A are widely expressed in various cell types, whereas BMPR1B shows more restricted expression (Derynck and Miyazono, 2008; Ebisawa et al., 1999; Miyazono et al., 2010; Nishitoh et al., 1996; ten Dijke et al., 1994). It was previously described that BMPR1B is endogenously expressed in LβT2 cells (Lee et al., 2007; Nicol et al., 2008). The structure of BMPR1A and BMPR1B are highly similar. Moreover, BMP2 and BMP4 bind to BMPR1A and BMPR1B with higher affinity than to ACVR1 (Derynck and Miyazono, 2008; Miyazono et al., 2010; ten Dijke et al., 1994). It is therefore interesting that BMPR1A is uniquely required for BMP2 induction of *Id3* transcription in LβT2 cells, and that its loss cannot be compensated for by BMPR1B. Perhaps the latter is expressed at too low a level to functionally compensate (Faure et al., 2005; Otsuka and Shimasaki, 2002) or the two receptors may function distinctly in this context (Ho and Bernard, 2009; Liu et al., 2003; Miura et al., 2010).

BMPs induce *Id1/ID1* expression through the activation of SMADs 1, 5, and 4 (Chen et al., 2006; Katagiri et al., 2002; Lopez-Rovira et al., 2002; Tian et al., 2010; Valdimarsdottir et al., 2002; Yang et al., 2010). Our data similarly implicate these SMADs in BMP2 induction of murine *Id3* in gonadotropes, whereas BMP4 induction of human *ID3* in vascular smooth muscle cells and ovarian cancer cells is also SMAD1/5/4-dependent (Davis et al., 2008; Shepherd et al., 2008). Interestingly, the loss of SMAD1 in gonadotropes cannot be fully compensated for by SMAD5, and vice versa, suggesting that the two SMADs may assume different roles in *Id3* regulation and/or that heteromers of SMAD1/5/4 may be most effective in stimulating *Id3* transcription.

Several BMP responsive elements have been described in the *Id1/ID1* promoter. One study identified a GC-rich region between −985/−957 of human *ID1* promoter as a necessary BRE (Katagiri et al., 2002). A second study identified the same GC-rich element and three additional CAGAC boxes as *cis*-elements required for BMP2-mediated induction of a human *ID1* reporter. Both sites were observed to bind SMAD1/4 and are located between −1046/−863 of the *ID1* promoter (Lopez-Rovira et al., 2002). In the murine *Id1* promoter, however, the BRE was localized between −1133/−1025

(Korchynskyi and ten Dijke, 2002). This region also contains a GC-rich BRE, specifically the GGCGCC palindrome, for binding SMAD5/4, and two CAGA(C) boxes for binding SMAD4. In contrast to the case for *Id1*/*ID1*, where there may be species diversity in BMP regulatory mechanisms, we identified a conserved BRE in the proximal murine *Id3* promoter that is physically and functionally conserved in the human *ID3* gene.

Specifically, we identified a 6-bp element critical for BMP2-mediated *Id3* transcription. Though a specific protein complex was shown to bind this site, its binding was BMP2-independent and its constituents remain undetermined. Furthermore, we were unable to demonstrate SMAD1 or SMAD5 as members of this complex, even though SMAD5/4 induction of *Id3* transcription is dependent upon this *cis*-element. Collectively, the data suggest that SMAD1/5 require the promoter element at −519/−514 (hereafter proximal BRE) to mediate the BMP2 response and that SMADs may produce their effects via protein–protein interaction rather than via direct DNA binding.

Though we identified this novel BRE, AAGATA, in the proximal murine *Id3* promoter, Shepherd et al. recently reported that BMP4 stimulates human *ID3* transcription in ovarian cancer cells via a more distal BRE, TGGCGCC, in the human *ID3* promoter (Shepherd et al., 2008). This observation led us to examine whether this latter element is also necessary for BMP2 induction of the murine *Id3* gene in gonadotropes. Indeed, the distal BRE is conserved in the murine *Id3* promoter and strongly contributes to BMP2 induction of both the human and murine *ID3*/*Id3* reporters. As indicated above, the proximal BRE in the murine *Id3* promoter is conserved in the proximal human *ID3* promoter and contributes to BMP2 induction of both the human and murine *ID3*/*Id3* reporters. When the two BREs were mutated in combination, the reduction in BMP2 activity was synergistic, suggesting that the proximal and distal BREs may cooperate in the regulation of human and murine *ID3*/*Id3* transcription. Shepherd et al. demonstrated the direct binding of SMAD1/5/4 to the distal BRE of the human *ID3* promoter; here we also show that SMAD1 binds to the distal BRE of the murine *Id3* promoter. At present, similar efforts have been unsuccessful in demonstrating SMAD1 binding to the proximal BRE.

BMPs regulate target gene expression through SMAD1/5/8 binding to GC-rich BREs, including GCCG elements (Ishida et al., 2000; Kim et al., 1997; Kusanagi et al., 2000; Xu et al., 1998) and 'bipartite elements', which are composed of the consensus sequence TGGCGCC with so-called 'CAGAC boxes' found in close proximity (Karaulanov et al., 2004). These bipartite elements are conserved between many BMP target genes, including of all four of the *Id* genes (Karaulanov et al., 2004). The proximal and distal BREs are perfectly conserved across all of the examined mammalian *ID3*/*Id3* promoters, including human, mouse, rat, cow, chimpanzee, and dog (Fig. S8). Furthermore, a conserved CAGAC box is found in close proximity to both the proximal and distal BREs in all cases; hence, both the proximal and distal BREs are potential bipartite elements. It has been proposed that BMP-regulated SMADs bind to the BRE site, whereas the CAGAC box mediates SMAD4 binding (Karaulanov et al., 2004). Indeed, BMP2 and BMP4 activity was significantly reduced when mutations were made to either the distal BRE site or its accompanying CAGAC box (Shepherd et al., 2008 and data not shown). The CAGAC box within the proximal candidate bipartite element may also play a role in directing BMP2 responsiveness by facilitating the actions of SMAD1/5 on the BRE; however, we have not yet studied this possibility. In addition to the distal site, Shepherd et al. also identified another bipartite element within the second intron of the *ID3* gene. These two elements were found to independently and synergistically regulate BMP4 mediated *ID3* expression in ovarian cancer cells (Shepherd et al., 2008). Here, we identified a potential bipartite element in the proximal *ID3*/*Id3* promoter, suggesting the possibility for a three-way interaction between the distal, proximal, and intronic elements to cooperatively regulate *ID3*/*Id3* expression. The intronic BRE identified in the human *ID3* promoter is also conserved in all the examined mammalian species (not shown); however, the closely associated CAGAC box is only present in human and chimpanzee suggesting that conservation may be limited to primates.

BMP stimulated *Id3* expression was first identified in the NIH3T3 cells, a mouse embryonic fibroblast cell line (Barone et al., 1994; Christy et al., 1991). We confirmed that both the distal and proximal BREs described here also mediate BMP2 induction of *Id3* transcription in these cells. This suggests that BMP2 likely regulates *Id3* expression through a mechanism common to most cell types, where the proximal and distal BREs act cooperatively to regulate *Id3* transcription.

Though we also demonstrated that BMP2 stimulates immediate-early induction of *Id2* transcription, we were unable to determine the underlying mechanisms because the murine −1561/+15 *Id2*-luciferase reporter available to us was unresponsive to BMP2. Recent data suggest that this likely stemmed from the absence of critical regulatory sequence in this reporter. In C2C12 cells, a longer *Id2* promoter-reporter (−3000/+80) was induced by BMP6 via a bipartite element at approximately −2.7 kb relative to the transcription start site (Nakahiro et al., 2010). Whether the same *cis*-element mediates BMP2 induction of *Id2* in gonadotrope cells remains to be determined.

In summary, we have determined relevant signaling components BMP2 employs to regulate *Id3* transcription. We have also identified a novel BMP2 response element in the proximal *ID3*/*Id3* promoter, which functions cooperatively with a distal element to regulate human and murine *ID3*/*Id3* expression. The mode of BMP2-mediated *Id3* expression we described here is likely a general mechanism conserved across cell types and mammalian species. Results from the present study may also contribute a more complete understanding of mechanisms controlling FSH synthesis as *Id3* has been implicated in BMP2-induced *Fshb* transcription.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2010.10.019.

References

Abe, J., 2006. Bone morphogenetic protein (BMP) family, SMAD signaling and *Id* helix-loop-helix proteins in the vasculature: the continuous mystery of BMPs pleotropic effects. J. Mol. Cell Cardiol. 41, 4–7.

Allendorph, G.P., Vale, W.W., Choe, S., 2006. Structure of the ternary signaling complex of a TGF-β superfamily member. Proc. Natl. Acad. Sci. U.S.A. 103, 7643–7648.

Barone, M.V., Pepperkok, R., Peverali, F.A., Philipson, L., 1994. *Id* proteins control growth induction in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 91, 4985–4988.

Belaguli, N.S., Zhang, M., Rigi, M., Aftab, M., Berger, D.H., 2007. Cooperation between GATA4 and TGF-β signaling regulates intestinal epithelial gene expression. Am. J. Physiol. Gastrointest. Liver Physiol. 292, G1520–G1533.

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

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

Chen, X., Zankl, A., Niroomand, F., Liu, Z., Katus, H.A., Jahn, L., Tiefenbacher, C., 2006. Upregulation of *ID* protein by growth and differentiation factor 5 (GDF5) through

a smad-dependent and MAPK-independent pathway in HUVSMC. J. Mol. Cell Cardiol. 41, 26–33.
Christy, B.A., Sanders, L.K., Lau, L.F., Copeland, N.G., Jenkins, N.A., Nathans, D., 1991. An *Id*-related helix-loop-helix protein encoded by a growth factor-inducible gene. Proc. Natl. Acad. Sci. U.S.A. 88, 1815–1819.
Coppe, J.P., Smith, A.P., Desprez, P.Y., 2003. *Id* proteins in epithelial cells. Exp. Cell Res. 285, 131–145.
Davis, B.N., Hilyard, A.C., Lagna, G., Hata, A., 2008. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454, 56–61.
Derynck, R., Miyazono, K. (Eds.), 2008. The TGF-β Family. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 121–139.
Ebisawa, T., Tada, K., Kitajima, I., Tojo, K., Sampath, T.K., Kawabata, M., Miyazono, K., Imamura, T., 1999. Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J. Cell Sci. 112 (Pt 20), 3519–3527.
Faure, M.O., Nicol, L., Fabre, S., Fontaine, J., Mohoric, N., McNeilly, A., Taragnat, C., 2005. BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. J. Endocrinol. 186, 109–121.
Gilboa, L., Nohe, A., Geissendorfer, T., Sebald, W., Henis, Y.I., Knaus, P., 2000. Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors. Mol. Biol. Cell. 11, 1023–1035.
Ho, C.C., Bernard, D.J., 2009. Bone morphogenetic protein 2 signals via BMPR1A to regulate murine follicle-stimulating hormone beta subunit transcription. Biol. Reprod. 81, 133–141.
Ho, C.C., Bernard, D.J., 2010. Bone morphogenetic protein 2 acts via inhibitor of DNA binding proteins to synergistically regulate follicle-stimulating hormone β transcription with activin A. Endocrinology 151 (7), 3445–3453.
Hoodless, P.A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L., Wrana, J.L., 1996. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85, 489–500.
Hua, H., Zhang, Y.Q., Dabernat, S., Kritzik, M., Dietz, D., Sterling, L., Sarvetnick, N., 2006. BMP4 regulates pancreatic progenitor cell expansion through *Id2*. J. Biol. Chem. 281, 13574–13580.
Huang, H.J., Wu, J.C., Su, P., Zhirnov, O., Miller, W.L., 2001. A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology 142, 2275–2283.
Inman, G.J., Nicolas, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., Hill, C.S., 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4 ALK5, and ALK7. Mol. Pharmacol. 62, 65–74.
Ishida, W., Hamamoto, T., Kusanagi, K., Yagi, K., Kawabata, M., Takehara, K., Sampath, T.K., Kato, M., Miyazono, K., 2000. Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. J. Biol. Chem. 275, 6075–6079.
Kaneko, K., Li, X., Zhang, X., Lamberti, J.J., Jamieson, S.W., Thistlethwaite, P.A., 2008. Endothelial expression of bone morphogenetic protein receptor type 1a is required for atrioventricular valve formation. Ann. Thorac. Surg. 85, 2090–2098.
Karaulanov, E., Knochel, W., Niehrs, C., 2004. Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. EMBO J. 23, 844–856.
Karaya, K., Mori, S., Kimoto, H., Shima, Y., Tsuji, Y., Kurooka, H., Akira, S., Yokota, Y., 2005. Regulation of *Id2* expression by CCAAT/enhancer binding protein beta. Nucleic Acids Res. 33, 1924–1934.
Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A., Suda, T., 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell Biol. 127, 1755–1766.
Katagiri, T., Imada, M., Yanai, T., Suda, T., Takahashi, N., Kamijo, R., 2002. Identification of a BMP-responsive element in *Id1*, the gene for inhibition of myogenesis. Genes Cells 7, 949–960.
Kim, J., Johnson, K., Chen, H.J., Carroll, S., Laughon, A., 1997. Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. Nature 388, 304–308.
Korchynskyi, O., ten Dijke, P., 2002. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the *Id1* promoter. J. Biol. Chem. 277, 4883–4891.
Kusanagi, K., Inoue, H., Ishidou, Y., Mishima, H.K., Kawabata, M., Miyazono, K., 2000. Characterization of a bone morphogenetic protein-responsive Smad-binding element. Mol. Biol. Cell. 11, 555–565.
Lamba, P., Santos, M.M., Philips, D.P., Bernard, D.J., 2006. Acute regulation of murine follicle-stimulating hormone beta subunit transcription by activin A. J. Mol. Endocrinol. 36, 201–220.
Lee, K.B., Khivansara, V., Santos, M.M., Lamba, P., Yuen, T., Sealfon, S.C., Bernard, D.J., 2007. Bone morphogenetic protein 2 and activin A synergistically stimulate follicle-stimulating hormone beta subunit transcription. J. Mol. Endocrinol. 38, 315–330.
Liu, J., Wilson, S., Reh, T., 2003. BMP receptor 1b is required for axon guidance and cell survival in the developing retina. Dev. Biol. 256, 34–48.
Lopez-Rovira, T., Chalaux, E., Massague, J., Rosa, J.L., Ventura, F., 2002. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of *Id1* gene. J. Biol. Chem. 277, 3176–3185.
Massague, J., Seoane, J., Wotton, D., 2005. Smad transcription factors. Genes Dev. 19, 2783–2810.
Mishina, Y., Hanks, M.C., Miura, S., Tallquist, M.D., Behringer, R.R., 2002. Generation of *Bmpr*/Alk3 conditional knockout mice. Genesis 32, 69–72.
Miura, S., Singh, A.P., Mishina, Y., 2010. *Bmpr1a* is required for proper migration of the AVE through regulation of Dkk1 expression in the pre-streak mouse embryo. Dev. Biol. 341, 246–254.
Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., Miyazono, K., 2002. Two major Smad pathways in TGF-β superfamily signalling. Genes Cells 7, 1191–1204.
Miyazono, K., 1999. Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins. Bone 25, 91–93.
Miyazono, K., Miyazawa, K., 2002. *Id*: a target of BMP signaling. Sci. STKE 2002, pe40.
Miyazono, K., Kamiya, Y., Morikawa, M., 2010. Bone morphogenetic protein receptors and signal transduction. J. Biochem. 147, 35–51.
Nakahiro, T., Kurooka, H., Mori, K., Sano, K., Yokota, Y., 2010. Identification of BMP responsive elements in the mouse *Id2* gene. Biochem. Biophys. Res. Commun. 399 (3), 416–421.
Nicol, L., Faure, M.O., McNeilly, J.R., Fontaine, J., Taragnat, C., McNeilly, A.S., 2008. Bone morphogenetic protein-4 interacts with activin and GnRH to modulate gonadotrophin secretion in LβT2 gonadotrophs. J. Endocrinol. 196, 497–507.
Nishitoh, H., Ichijo, H., Kimura, M., Matsumoto, T., Makishima, F., Yamaguchi, A., Yamashita, H., Enomoto, S., Miyazono, K., 1996. Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. J. Biol. Chem. 271, 21345–21352.
Nohe, A., Hassel, S., Ehrlich, M., Neubauer, F., Sebald, W., Henis, Y.I., Knaus, P., 2002. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J. Biol. Chem. 277, 5330–5338.
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.
Peng, Y., Kang, Q., Luo, Q., Jiang, W., Si, W., Liu, B.A., Luu, H.H., Park, J.K., Li, X., Luo, J., Montag, A.G., Haydon, R.C., He, T.C., 2004. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. J. Biol. Chem. 279, 32941–32949.
Samanta, J., Kessler, J.A., 2004. Interactions between *ID* and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. Development 131, 4131–4142.
Shepherd, T.G., Theriault, B.L., Nachtigal, M.W., 2008. Autocrine BMP4 signalling regulates *ID3* proto-oncogene expression in human ovarian cancer cells. Gene 414, 95–105.
Shimasaki, S., Moore, R.K., Otsuka, F., Erickson, G.F., 2004. The bone morphogenetic protein system in mammalian reproduction. Endocr. Rev. 25, 72–101.
Sieber, C., Kopf, J., Hiepen, C., Knaus, P., 2009. Recent advances in BMP receptor signaling. Cytokine Growth Factor Rev. 20, 343–355.
ten Dijke, P., Yamashita, H., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C.H., Miyazono, K., 1994. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J. Biol. Chem. 269, 16985–16988.
Therrien, M., Drouin, J., 1993. Cell-specific helix-loop-helix factor required for pituitary expression of the pro-opiomelanocortin gene. Mol. Cell Biol. 13, 2342–2353.
Tian, F., Zhou, A.X., Smits, A.M., Larsson, E., Goumans, M.J., Heldin, C.H., Boren, J., Akyurek, L.M., 2010. Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro. Biochem. Biophys. Res. Commun. 392, 283–288.
Urist, M.R., 1965. Bone: formation by autoinduction. Science 150, 893–899.
Valdimarsdottir, G., Goumans, M.J., Rosendahl, A., Brugman, M., Itoh, S., Lebrin, F., Sideras, P., ten Dijke, P., 2002. Stimulation of *Id1* expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation 106, 2263–2270.
Vinals, F., Reiriz, J., Ambrosio, S., Bartrons, R., Rosa, J.L., Ventura, F., 2004. BMP-2 decreases Mash1 stability by increasing *Id1* expression. EMBO J. 23, 3527–3537.
Xu, X., Yin, Z., Hudson, J.B., Ferguson, E.L., Frasch, M., 1998. Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm. Genes Dev. 12, 2354–2370.
Yang, J., Davies, R.J., Southwood, M., Long, L., Yang, X., Sobolewski, A., Upton, P.D., Trembath, R.C., Morrell, N.W., 2008. Mutations in bone morphogenetic protein type II receptor cause dysregulation of *Id* gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. Circ. Res. 102, 1212–1221.
Yang, J., Li, X., Al-Lamki, R.S., Southwood, M., Zhao, J., Lever, A.M., Grimminger, F., Schermuly, R.T., Morrell, N.W., 2010. Smad-dependent and Smad-independent induction of *Id1* by prostacyclin analogues inhibits proliferation of pulmonary artery smooth muscle cells in vitro and in vivo. Circ. Res. 107, 252–262.
Yeh, K., Lim, R.W., 2000. Genomic organization and promoter analysis of the murine *Id3* gene. Gene 254, 163–171.
Yu, P.B., Beppu, H., Kawai, N., Li, E., Bloch, K.D., 2005. Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. J. Biol. Chem. 280, 24443–24450.
Yu, P.B., Deng, D.Y., Beppu, H., Hong, C.C., Lai, C., Hoyng, S.A., Kawai, N., Bloch, K.D., 2008. Bone morphogenetic protein (BMP) type II receptor is required for BMP-mediated growth arrest and differentiation in pulmonary artery smooth muscle cells. J. Biol. Chem. 283, 3877–3888.