# Regulation of FSHβ induction in LβT2 cells by BMP2 and an Activin A/BMP2 chimera, AB215

**Jae Woo Jung[1,†], Chihoon Ahn[1], Sun Young Shim[1], Peter C Gray[3], Witek Kwiatkowski[1,2] and Senyon Choe[1,2]**

[1]Joint Center for Biosciences, Songdo Global University Campus, 187 Songdo-dong, Yeonsu-gu, Incheon 406-840, Korea
[2]Structural Biology Laboratory and [3]Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, USA
[†]J W Jung is now at Interdisciplinary Graduate Program in Genetic Engineering, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul, Korea

Correspondence should be addressed to S Choe
**Email**
choe@salk.edu

## Abstract

Activins and bone morphogenetic proteins (BMPs) share activin type 2 signaling receptors but utilize different type 1 receptors and Smads. We designed AB215, a potent BMP2-like Activin A/BMP2 chimera incorporating the high-affinity type 2 receptor-binding epitope of Activin A. In this study, we compare the signaling properties of AB215 and BMP2 in HEK293T cells and gonadotroph LβT2 cells in which Activin A and BMP2 synergistically induce FSHβ. In HEK293T cells, AB215 is more potent than BMP2 and competitively blocks Activin A signaling, while BMP2 has a partial blocking activity. Activin A signaling is insensitive to BMP pathway antagonism in HEK293T cells but is strongly inhibited by constitutively active (CA) BMP type 1 receptors. By contrast, the potencies of AB215 and BMP2 are indistinguishable in LβT2 cells and although AB215 blocks Activin A signaling, BMP2 has no inhibitory effect. Unlike HEK293T, Activin A signaling is strongly inhibited by BMP pathway antagonism in LβT2 cells but is largely unaffected by CA BMP type 1 receptors. BMP2 increases phospho-Smad3 levels in LβT2 cells, in both the absence and the presence of Activin A treatment, and augments Activin A-induced FSHβ. AB215 has the opposite effect and sharply decreases basal phospho-Smad3 levels and blocks Smad2 phosphorylation and FSHβ induction resulting from Activin A treatment. These findings together demonstrate that while AB215 activates the BMP pathway, it has opposing effects to those of BMP2 on FSHβ induction in LβT2 cells apparently due to its ability to block Activin A signaling.

**Key Words**
- transforming growth factor beta (TGFβ)
- activin
- bone morphogenetic protein (BMP)
- protein structure
- signal transduction

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## Introduction

Follicle-stimulating hormone (FSH) is a gonadotrophin that is synthesized and secreted by gonadotroph cells of the anterior pituitary gland. Functionally, FSH is a glycoprotein heterodimer consisting of a β subunit specific to FSH, FSHβ, and an α subunit, chorionic gonadotrophin alpha (CGA, also known as α-glycoprotein subunit) that is shared with luteinizing hormone (LH), thyroid-stimulating hormone, and human chorionic gonadotrophin. FSH stimulates ovarian follicle growth and maturation in female mammals and disruption of its signaling leads to abnormal oogenesis (Dierich *et al.* 1998). Clinically, *FSHB* (for FSHβ subunit) or *FSHR* (for FSH receptor) gene mutations cause primary

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(Doherty *et al.* 2002, Kottler *et al.* 2010) or secondary (Beau *et al.* 1998) amenorrhea by premature follicle development at the pre-antral stage. Low FSH levels are also observed in polycystic ovarian syndrome (Banaszewska *et al.* 2003) and Kallmann syndrome (Bouvattier *et al.* 2012). Furthermore, uncontrolled FSH secretion, resulting in high FSH levels, was also observed in serious reproductive diseases such as premature menopause (Ahmed Ebbiary *et al.* 1994), gonadal dysgenesis (Breckwoldt *et al.* 1980), and infertility. FSH is also essential for male reproduction and plays a key role in regulating spermatogenesis (Elkington & Blackshaw 1974, Matsumoto *et al.* 1986, Kerr *et al.* 1992, Lerchl *et al.* 1993). Therefore, precise regulation of FSH secretion is fundamental for human reproduction and its dysregulation can cause serious illnesses.

FSH production is closely correlated with the production of the FSHβ subunit. There is a relative abundance of CGA compared with FSHβ (Bernard *et al.* 2010) and *FSHB* mRNA levels are synchronized with circulating FSH hormone levels (Ortolano *et al.* 1988, Halvorson *et al.* 1994). FSHβ is produced from the *FSHB* gene in gonadotroph cells by stimulation from gonadotrophin-releasing hormone (GnRH) and Activin A. GnRH is produced in the hypothalamus and secreted into the pituitary portal vasculature in a pulsatile fashion. GnRH triggers the secretion of both LH and FSH (Belchetz *et al.* 1978, Southworth *et al.* 1991) and the frequency of GnRH pulses has been considered to be the determinant of which, of these two hormones, is preferentially targeted for secretion (Marshall *et al.* 1993, Ferris & Shupnik 2006). Activin A, a member of the transforming growth factor beta (TGFβ) superfamily, was discovered for its ability to stimulate the production of FSH but not LH in rat primary pituitary cultures (Vale *et al.* 1988, Ying 1988, Bilezikjian *et al.* 2004). FSH levels surge twice during the female estrous cycle. The first surge occurs during the late follicular phase and is induced by GnRH (Ortolano *et al.* 1988, Halvorson *et al.* 1994). The second surge occurs in the luteal phase and is considered to be dependent on Activin A (DePaolo *et al.* 1979, Hoak & Schwartz 1980).

Activin A is a ubiquitous regulator of myriad cellular functions during development and in the adult animal. It plays roles in cell proliferation (Boitani *et al.* 1995, Ota *et al.* 2003, Mendis *et al.* 2011), differentiation (Seishima *et al.* 1999, Sulzbacher *et al.* 2009), apoptosis (Zhang *et al.* 1997, Chen *et al.* 2000), immune responses (Jones *et al.* 2007, Robson *et al.* 2009), and many other cellular activities. Activin A is a homodimer of βA subunits and signals by binding to types 1 and 2 transmembrane serine kinase receptors. High-affinity binding of Activin A to one of its type 2 receptors (ActR2 or ActR2b) allows for subsequent recruitment of its type 1 receptor Alk4 to form an active signaling complex. In this complex, the constitutively active (CA) type 2 receptors phosphorylate the type 1 receptors, thereby activating the type 1 receptor kinases which in turn phosphorylate and activate cytoplasmic SMAD2 and SMAD3 proteins. Upon phosphorylation, SMAD2 and SMAD3 form complexes with SMAD4 and are translocated into the nucleus where they regulate transcription of target genes in a complex and cell type-specific manner. This signaling cascade is regulated by feedback inhibitors of Activin A such as follistatin, which contributes to the precise regulation of FSH production in gonadotrophs.

Bone morphogenetic protein 2 (BMP2) is another member of the TGFβ superfamily that is well known for regulating osteogenesis (Wozney *et al.* 1988) and chondrogenesis (Yoon & Lyons 2004) via activation of the SMAD1/5/8 signaling pathway. BMP2 signals by binding to its type 1 receptors, BMPR1a (ALK3), BMPR1b (ALK6), or ActR1a (ALK2), and recruiting its type 2 receptors, i.e. ActR2, ActR2b, or BMPR2. BMP2 triggers type 1 receptor activation and phosphorylation of SMAD1/5/8, which, similar to SMAD2/3, binds SMAD4 and translocates into the nucleus where they regulate specific target genes. Although BMP2 and Activin A signal via different SMAD proteins, their coexistence in some tissues such as perinatal sensory neuron target tissues (Hall *et al.* 2002) and pituitary suggests some crosstalk and possible synergy between the two pathways. Indeed, BMP2 is involved in pituitary development (Ericson *et al.* 1998) and also expressed in adult murine pituitary (Lee *et al.* 2007). BMP2 regulates FSHβ synthesis in the murine gonadotroph cell line, LβT2, and can act synergistically with Activin A (Lee *et al.* 2007, Ho & Bernard 2010). The synergistic effect of BMP2 and Activin A was proposed to be due to BMP2 induction of inhibitor of DNA-binding (ID) proteins ID2 and ID3 via SMAD3 (Ho & Bernard 2010). However, it has recently been shown that BMP2 also activates the SMAD2/3 pathway noncanonically through ALK3 (Wang *et al.* 2014).

As BMP2 and Activin A both bind the type 2 receptors ActR2 and ActR2b in almost exactly the same spatial configuration (Greenwald *et al.* 2004, Allendorph *et al.* 2006), we hypothesized that we could replace the low-affinity type 2 binding epitope of BMP2 with the high-affinity type 2 receptor-binding epitope of Activin A. Our prediction was that such a swap would yield a chimeric ligand with enhanced type 2 receptor affinity and signaling potency that retains BMP2-like type 1 receptor utilization and SMAD signaling specificity. Indeed, one such ligand,



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AB215, utilizes the same signaling receptors and Smads as BMP2 but possesses signaling activity and potency that are enhanced relative to BMP2 as demonstrated in *in vitro* signaling assays (Allendorph *et al.* 2011). In this study, we sought to determine whether AB215 functions synergistically with Activin A in the regulation of the FSHβ production in a manner resembling BMP2. Our results indicate that AB215 and BMP2 have opposing effects on Activin A-induced FSHβ induction and that rather than synergizing with Activin A, AB215 competitively blocks Activin A signaling apparently via competition for activin type 2 receptor binding. Overall, our data demonstrate that the potencies of AB215 and BMP2 and their effects on the Activin pathway can vary dramatically in a cell type-specific manner.

## Materials and methods

### Reagents

BMP2 was purchased from joint Protein Central (http://jointproteincentral.com). Activin A was purchased from R&D systems (http://www.rndsystems.com). AB204 and AB215 were prepared as described previously (Allendorph *et al.* 2011). In brief, Activin A/BMP2 (AB2) chimera library is a synthetic assortment of six sequence segments originating from two parental molecules, and AB215 is a member of AB2 chimera library. AB215 were expressed in *Escherichia coli* and chemically refolded. After the purification steps of heparin affinity and C4 reverse-phase chromatography using HPLC, proteins were lyophilized. Before use, the lyophilized proteins were reconstituted in 1 mM hydrochloric acid (HCl) before diluting by at least a factor of 100 in a relevant final buffer including PBS. LDN193189 (Selleck Chemicals, Houston, TX, USA) was reconstituted in DMSO at 10 mM and diluted in media before use.

### Cell culture

HEK293T cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and transformed mouse gonadotroph cell line, LβT2, originates from Dr Pamela Mellon (University of California, San Diego, CA, USA) (Alarid *et al.* 1996). Both cell lines were grown in 37 °C humidified atmosphere of 5% $CO_2$ in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and sub-cultured using 1:2 split every third day.

### Luciferase assay

Luciferase reporter assay driven by Activin-responsive element (ARE-lux), inhibitor of DNA binding 1 (ID1-luc), and −1990/+1 mFshb (mFSHβ-luc) promoters were performed in a 96-well plate in triplicates (three wells per point) and were repeated four times. Cells were co-transfected with pCMV-β-galactosidase (β-Gal) plasmid as a normalizing factor for the transfection efficiency. CA ALKs used were ALK2-Q207D (CA-ALK2), ALK3-Q233D (CA-ALK3), and ALK6-Q203D (CA-ALK6). For transfection, Fugene 6 (Promega, Madison, WI, USA) was used in 1:2 dilution of (DNA in μg, Fugene 6 in μl) dilution for HEK293T cells and 2:6 dilution for LβT2 cells. Signaling transducers (FAST1 or FAST2) and external signaling component (SMADs) were not amplified to observe the outcome in the natural state. The entire promoter assay was carried out with reverse transfection method in reduced serum Opti-MEM (Invitrogen). After 18 h of transfection, cells were treated with indicated reagents for 24 h. At the end of the incubation, cells were lysed using Luciferase lysis buffer (Promega) and luminescence was measured using a plate luminometer (Berthhold, Bad Wildbad, Germany).

### Western blot

Cells were plated in a 12-well plate (BD Biosciences, San Jose, CA, USA) at a density of $2\times10^5$ cells/well. Cells were treated as indicated and lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with 1 mM phenylmethylsulphonyl fluoride and phosphatase inhibitor cocktail (Roche). Cell lysate's total protein amount was quantified using Bradford assay. Whole cell lysates were separated on SDS–polyacrylamide gel and transferred onto nitrocellulose or PVDF membrane for standard western blot analysis. P-SMAD2 (cat. #3101) and 3 (cat. #9520) antibodies were purchased from Cell Signaling Technology, and β-actin from Sigma.

### Real-time PCR

Cells were plated in a 12-well plate (BD Biosciences) at a density of $1\times10^5$ cells/well and cultured in a medium supplemented with 10% FBS. After 16–24 h, cells were treated with indicated reagents for 48 h. After exposure, RNA was extracted with TRIsure (Bioline Taunton, MA, USA) according to the manufacturer's instruction. cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan) according to the manufacturer's instruction. Analysis of mRNA



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expression was determined with quantitative real-time PCR using Thunderbird SYBR qPCR mix (Toyobo) and 10 pM primers according to the manufacturer's instruction. The sequences of primers are as follows: mouse cyclophilin, sense: 5′-CAGACGCCACTGTCGCTTT-3′ and anti-sense: 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; mouse Fshβ, sense: 5′-CTCTGAAGAGCGTGGAGTATTG-3′ and anti-sense: 5′-GTTACTACCTCCTGTCCTGTCT-3′. Abundance of mRNA in each sample was determined by the differences between the cycle threshold (*C*t) values for each genes and cyclophilin, Δ*C*t. Relative ratios of mRNA expression levels were defined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$, which reflect changes in mRNA expression levels from treated cells compared with those from untreated cells. All experiments were carried out at least three times in triplicate.

## Results

### BMP2, AB215, and CA BMP type 1 receptors inhibit Activin A signaling via distinct mechanisms in HEK293T cells

We have previously created a library of Activin A/BMP2 (AB2) chimeras denoted by the code (BXXXXX), where X is either A (Activin A) or B (BMP2) (Allendorph *et al*. 2011). AB215 (BABBBA) was designed to have the majority of the type 2 receptor-binding epitope of Activin A (segments 2 and 6) and the majority of type 1 receptor-binding epitope of BMP2 (segments 1, 3, 4, and 5) (Fig. 1A). Consistent with our previous findings, using C2C12 cells (Allendorph *et al*. 2011), AB215 has a higher potency than BMP2 in activating a Smad1/5/8-responsive ID1-luciferase reporter in HEK293T cells (Fig. 1B). We employed the potent kinase inhibitor, LDN193189, to investigate a potential crosstalk between BMP and Activin signaling pathways. LDN193189 is an ATP-competitive inhibitor, which binds on the kinase hinge region of BMP type 1 receptors. We show that LDN193189 (150 nM) effectively blocks BMP2 and AB215 induction of the ID1-luciferase reporter in HEK293T cells (Fig. 1B) and we used this dose in all subsequent experiments where LDN193189 was used. Activin A induction of the Smad2/3-responsive ARE-Lux reporter was unaffected by LDN193189 in HEK293T cells (Fig. 1C), indicating that the Activin A response does not require basal activation of the BMP pathway. However, BMP2 partially diminishes Activin A signaling in HEK293T cells in a manner that is rescued to a modest extent by the BMP inhibitor LDN193189 (Fig. 1D). On the other hand, AB215 completely blocks Activin A signaling in HEK293T cells and this does not require BMP pathway activation as it is unaffected by LDN193189 treatment (Fig. 1E). To investigate the effects of downstream BMP pathway activation on Activin A signaling, we transfected CA forms of ALK2, ALK3, and ALK6 and monitored SMAD1/5/8 and SMAD2/3 signaling using ID1-luc and ARE-lux reporter assays respectively. Each of these CA BMP type 1 receptors significantly amplifies SMAD1/5/8 signaling as expected (Fig. 1F) but, at the same time, dramatically attenuates SMAD2/3 signaling (Fig. 1G).

### AB215 inhibits Activin A signaling in LβT2 cells, while BMP2 and Smad1/5/8 activation do not

In contrast to HEK293T cells (Fig. 1) and C2C12 cells (Allendorph *et al*. 2011), BMP2 and AB215 have similar potencies in LβT2 cells as measured by their ability to activate the ID1-luc reporter (Fig. 2A). As predicted, LDN193189 effectively blocks the signaling of both ligands in these cells (Fig. 2A). Also unlike what was observed in HEK293T cells, Activin A induction of the ARE-lux reporter is highly dependent on basal BMP signaling in LβT2 cells as it is substantially blocked by LDN193189 (Fig. 2B). Furthermore, and also differing from what we observe in HEK293T cells, BMP2 treatment over a range of doses has no inhibitory effect on Activin A induction of the ARE-lux reporter in LβT2 cells while blocking SMAD1/5/8 signaling again diminishes the Activin A response (Fig. 2C). However, AB215 completely and dose dependently blocks Activin A signaling in LβT2 cells, as in HEK293T cells, apparently by directly competing with Activin A for type 2 receptor binding (Fig. 2D). Finally, and in further contrast to what we observe in HEK293T cells, transfection of the CA BMP type 1 receptors had only a modest effect (CA-ALK2) or no effect (CA-ALK3 and CA-ALK6) on Activin A induction of ARE-lux in LβT2 cells (Fig. 2F).

### BMP2 and AB215 have opposing effects on Activin A induction of the *FSHB* promoter in L$\beta$T2 gonadotrophs

BMP2 and Activin A synergistically induce expression of FSHβ in gonadotroph LβT2 cells (Lee *et al*. 2007, Ho & Bernard 2010, Wang *et al*. 2014) and we compared the effects of BMP2 and AB215 on *FSHB* promoter activity. LβT2 cells express BMPs endogenously (Huang *et al*. 2001) and basal BMP signaling is required for full Activin A induction of *FSHB* promoter activity in these cells as indicated by the dramatic inhibitory effect of LDN193189 over a range of Activin A doses (Fig. 3A). We observe that while BMP2 dose dependently augments Activin A



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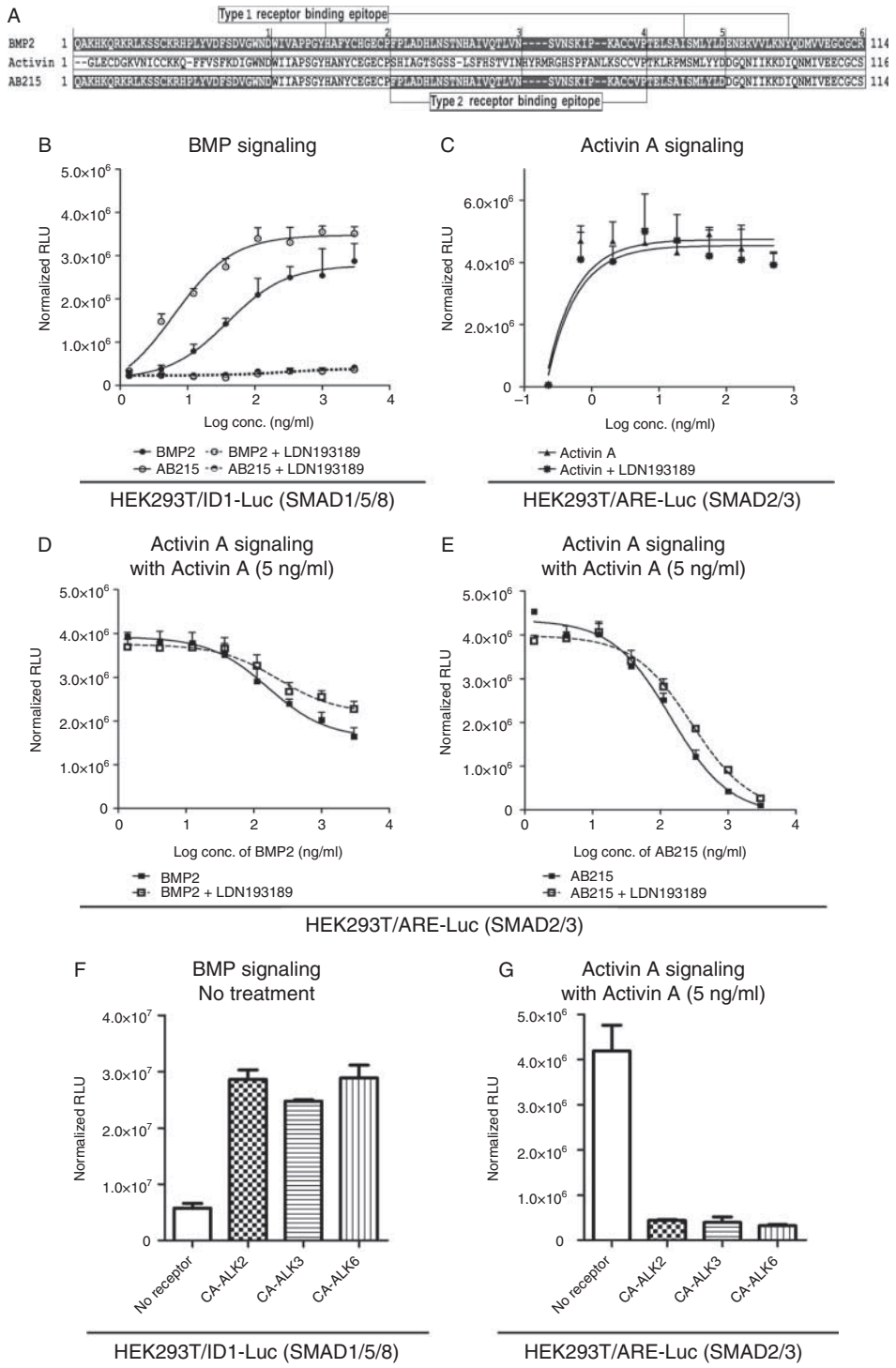


**Figure 1**

AB215 and BMP2 inhibit Activin A signaling to varying degrees in HEK293T cells. (A) The sequences of Activin A, BMP2, and AB215 are shown illustrating the segmental composition of AB215 and the location of the type 1 receptor- and type 2 receptor-binding epitopes. HEK293T cells were transfected with ID1-Luc (B and F), ARE-Lux (C, D, E and G), and constitutively active BMP type 1 receptors (CA-ALK2, CA-ALK3, or CA-ALK6) (F and G). Cells were treated with the indicated doses of BMP2, AB215, or Activin A (B, D, E and G) in the absence or presence of the ALK2/3/6 inhibitor LDN193189 (150 nM, dotted lines). All the assays were carried out in triplicate with five independent experiments and transfection differences were normalized using β-galactosidase. Curves were fitted using the Prism Software (GraphPad, San Diego, CA, USA) and shown in mean ± S.D. RLU, relative light units.

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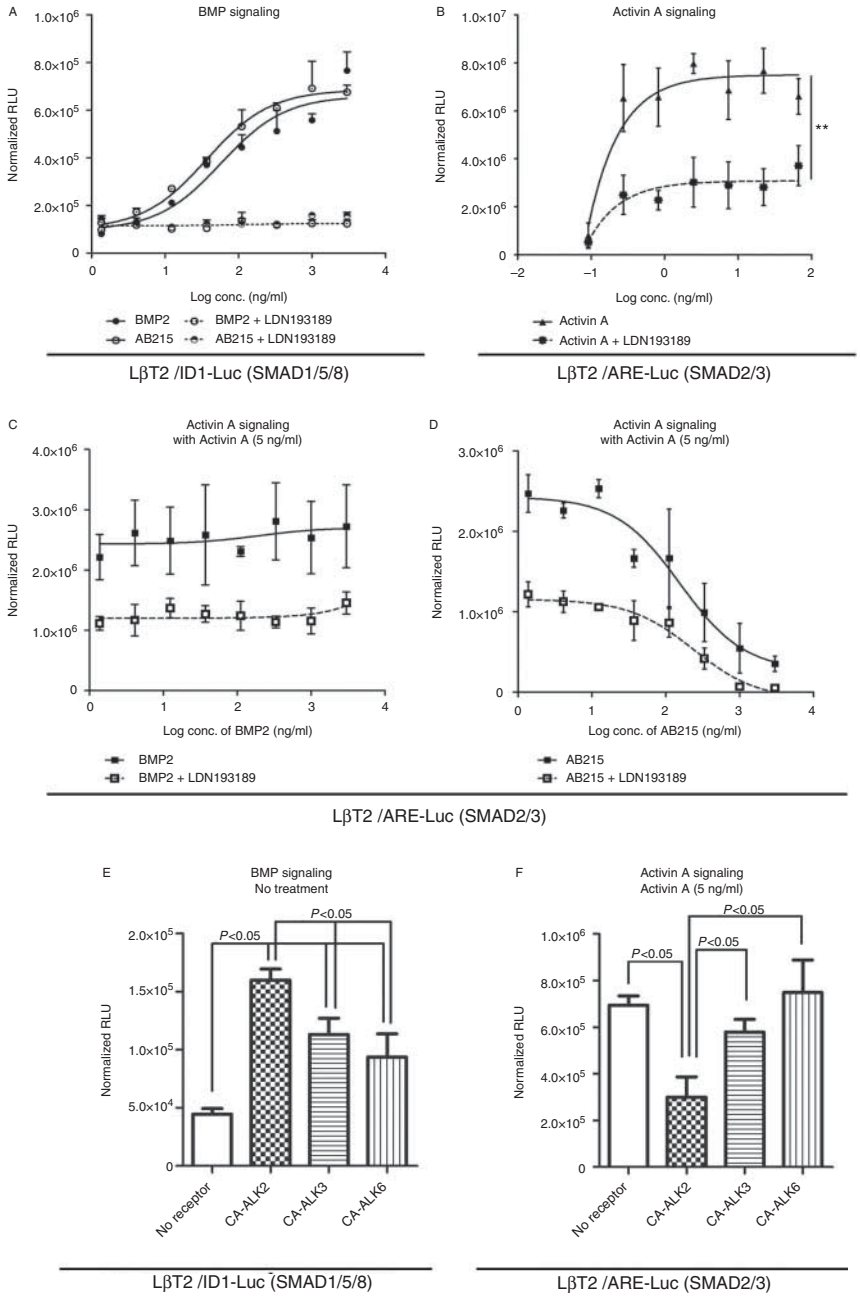


**Figure 2**

AB215 but not BMP2 inhibits Activin A signaling in LβT2 cells. HEK293T cells were transfected with ID1-Luc (A and E), ARE-Lux (B, C, D and F) and constitutively active BMP type 1 receptors (CA-ALK2, CA-ALK3, or CA-ALK6) (E and F), and treated with the indicated doses of BMP2, AB215, or Activin A (A, C, D and F) in the absence or presence of the ALK2/3/6 inhibitor LDN193189 (150 nM, dotted lines). All the assays were done in triplicate with five independent experiments and transfection differences were normalized using β-galactosidase. Curves were fitted using the Prism Software (GraphPad) and shown in mean ± S.D. RLU, relative light units. The *T*-test was employed in (B) and one-way ANOVA with Dunnett's *post hoc* test was employed in (E and F).

induction of *FSHB* promoter activity (Fig. 3B), AB215 has the opposite effect and inhibits the Activin A induction of FSHB (Fig. 3C). Moreover, blocking SMAD1/5/8 signaling with LDN193189 results in loss of BMP2's augmentation of the Activin A induction of *FSHB* promoter activity but has little or no effect on AB215 inhibition of the Activin A response (Fig. 3B and C).

The mechanistic basis for the synergy between BMP2 and Activin A in LβT2 cells is not fully understood but was reported to involve Smad2 and Smad3 and more recently

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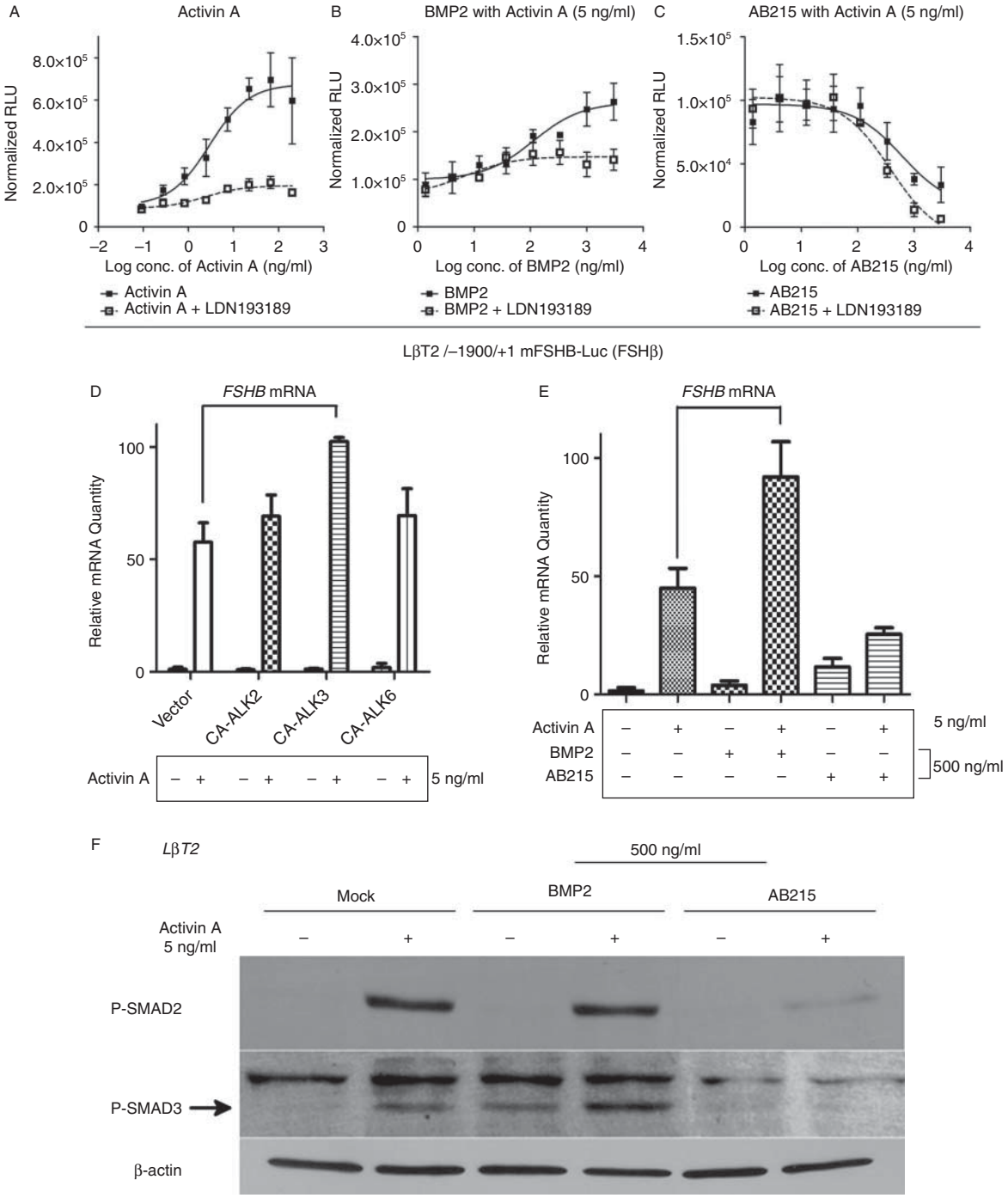


**Figure 3**

AB215 and BMP2 have opposing effects on Activin A induction of FSHβ. LβT2 cells were transfected with the −1900/+1 mFSHB-Luc construct and treated with the indicated doses of AB215, BMP2, and Activin A in the absence or presence of LDN193189 (150 nM, dotted lines; A, B and C). All the assays were carried out in triplicate with five independent experiments, and transfection differences were normalized using β-galactosidase. Curves were fitted using the Prism Software and shown in mean±S.D. RLU, relative light unites. Alternatively, *FSHB* mRNA levels were measured by RT-PCR after transfecting LβT2 cells with CA-ALK2, CA-ALK3, or CA-ALK6 and treating them as indicated with Activin A (D) or after treating untransfected cells with Activin A, BMP2, and/or AB215 as indicated (E). LβT2 cells were also treated with Activin A, BMP2, and/or AB215 as indicated and the resulting cell lysates were analyzed for the presence of phospho-Smad2 and phospho-Smad3 (F). Significance has been calculated using one-way ANOVA with Dunnett's (D) and Tukey's (E) *post hoc* tests and data are shown in means±S.D.

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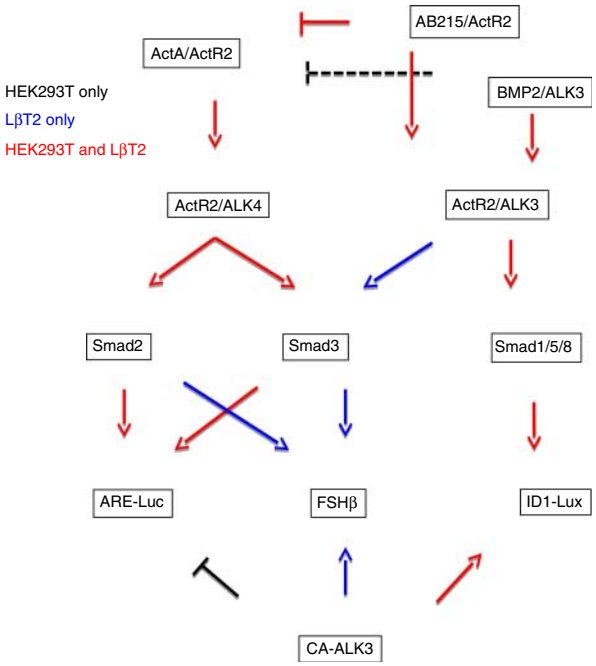


**Figure 4**
AB215 and BMP2 signaling in HEK293T cells and LβT2 cells. Diagram illustrating the signaling and crosstalk of Activin A, AB215, and BMP2 in HEK293T cells and LβT2 cells. BMP pathway activation inhibits Activin A signaling in HEK293T cells but either has no inhibitory effect (ARE-Lux induction) or enhances (FSHβ induction) Activin A signaling in LβT2 cells. AB215 inhibits Activin A signaling in both cell lines while BMP2 partially inhibits Activin A signaling in HEK293T cells but not in LβT2 cells. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-14-0317.

BMP signaling via ALK3 (Wang *et al*. 2014). Consistently, we observe that CA-ALK3, but not CA-ALK2 or CA-ALK6, increases *FSHB* mRNA levels in LβT2 cells (Fig. 3D). We further find that, while BMP2 treatment alone has no detectable effect on FSHβ expression, BMP2 and Activin A together result in nearly twice as much *FSHB* mRNA production as that produced by Activin A alone (Fig. 3E). By contrast, AB215 alone has little effect on *FSHB* mRNA levels but inhibits Activin A induction of FSHβ (Fig. 3E). Western blot analysis shows that Activin A treatment triggers phosphorylation of Smad2 and Smad3 in LβT2 cells as expected (Fig. 3F). We further find that BMP2 treatment alone triggers Smad3 phosphorylation and augments Smad3 phosphorylation resulting from Activin A treatment, but that it does not affect Smad2 phosphorylation levels in the absence or presence of Activin A (Fig. 3F). By contrast, AB215 reduces basal phospho-Smad3 levels and blocks phosphorylation of Smad2 and Smad3 caused by Activin A treatment (Fig. 3F). Thus, BMP2 and AB215 have opposing effects on Activin A signaling via Smad2 and Smad3, providing an explanation for the opposing effects of these ligands on FSHβ regulation in LβT2 gonadotrophs.

## Discussion

BMPs and Activins are both expressed endogenously in gonadotrophs. However, modulation of *FSHB* transcription by BMPs varies between species (Visser & Themmen 2014). In murine primary pituitary cells, various BMPs including BMP15, BMP7, BMP6, and BMP4 stimulate *FSHB* transcription (Huang *et al*. 2001, Otsuka & Shimasaki 2002, Nicol *et al*. 2008), whereas BMP6 and BMP4 inhibit *FSHB* transcription in ovine cells (Faure *et al*. 2005). In accordance with the stimulatory effect of BMPs in murine primary pituitary cells, it has been shown that BMP2 acts synergistically with Activin A to regulate FSHβ expression in LβT2 cells (Lee *et al*. 2007, Wang *et al*. 2014). BMP2 has been previously reported to induce non-canonical phosphorylation of Smad3 (Holtzhausen *et al*. 2014, Wang *et al*. 2014) consistent with our results showing that BMP2 triggers Smad3 phosphorylation in LβT2 cells (Fig. 3F). However, such non-canonical BMP2 signaling is clearly cell type specific, as the ALK2/3/6 inhibitor, LDB193189, severely inhibits Activin A signaling in LβT2 cells (Fig. 2B) but has no effect on Activin A signaling in HEK293T cells (Fig. 1C). While it has also been reported that BMP2 induces SMAD2 phosphorylation in LβT2 cells (Wang *et al*. 2014), we did not observe this effect (Fig. 3F). However, our results agree with previous results showing that ALK3, and not ALK2 or ALK6, is responsible for BMP2-induced Smad3 phosphorylation and subsequent augmentation of Activin-induced FSH production (Fig. 3D and F). Our results confirm other reports showing that SMAD3 is the predominant SMAD mediating Activin A induction factor of FSHβ synthesis (Wang *et al*. 2014). Additionally, the results of SMAD depletion studies (Bernard *et al*. 2010) have shown that SMAD3 but not SMAD2 is the main regulator of FSHβ expression. However, our data suggest that SMAD3 phosphorylation by itself is insufficient to fully induce FSHβ expression, as BMP2 alone causes SMAD3 phosphorylation but is unable to up-regulate FSHβ expression noticeably (Fig. 3E).

In an effort to find new methods of regulating FSHβ expression, we tested a BMP2/Activin A chimera, AB215, with type 1 receptor-binding properties and SMAD signaling specificity of BMP2, but an enhanced potency resulting from high-affinity type 2 receptor binding resembling that of Activin A (Allendorph *et al*. 2011). We have previously demonstrated that AB215 has a higher potency than BMP2 in preosteoblastic C2C12 cells



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(Allendorph *et al*. 2011) and herein we demonstrate that the same holds true in HEK293T cells (Fig. 1B). It is therefore intriguing to observe that the potencies of AB215 and BMP2 are indistinguishable in LβT2 cells (Fig. 1C). Generally, the potency of TGFβ superfamily ligands corresponds to their affinity for their higher affinity receptor type (Qian *et al*. 1996). AB215 has a higher affinity for activin type 2 receptors than for BMP type 1 receptors, while BMP2 has the opposite receptor preference (Allendorph *et al*. 2011). As AB215 also has a higher affinity for activin type 2 receptors than BMP2 has for its type 1 receptors (Allendorph *et al*. 2011), it follows that AB215 should have a higher overall potency than BMP2, and this is indeed what we observe in HEK293T and C2C12 cells. However, this argument is predicted to be true only when the number and distribution of type 1 and type 2 receptors are similar. If BMP type 1 receptors are sparse relative to activin type 2 receptors, then the potencies of BMP2 and AB215 can be similar as is observed in LβT2 cells. In such a situation, rapid binding of AB215 to activin type 2 receptors would be followed by slow binding to rare BMP type 1 receptors, while rapid binding of BMP2 to limiting type 1 receptors would be followed by relative rapid recruitment of abundant type 2 receptors (Fig. 4).

It was initially surprising to find that AB215 does not mimic the ability of BMP2 to synergize with Activin A in promoting FSH expression in LβT2 cells (Fig. 3B), but rather inhibits Activin A-induced FSH production (Fig. 3C). It is, however, very clear from our results that, in the context of the cooperative signaling between BMP and Activin pathways, AB215 is a potent Activin A antagonist through direct competition for activin type 2 receptor binding (Figs 1E, 2D and 3F). An inhibitory effect of BMP2 on Activin A signaling is also consistent with competition between these two ligands for type 2 receptors in HEK293T cells (Fig. 1D). Unlike AB215, however, BMP2 only partially blocks Activin A signaling in these cells even at the highest BMP2 dose (3 mg/ml), suggesting that BMP2 antagonism of Activin A signaling requires a factor that is limiting. BMP2 assembles its signaling receptors by first binding to its higher affinity type 1 receptors and then recruiting its lower affinity type 2 receptors. Therefore, BMP type 1 receptors may be limiting in HEK293T cells such that only a fraction of the type 2 receptors can be sequestered by BMP2, thereby preventing BMP2 from completely blocking Activin A signaling (Fig. 1D). We also note that blocking BMP signaling with an inhibitor of ALK2/3/6 kinases partially alleviates the inhibitory effect of BMP2 on Activin A signaling in HEK293T cells, suggesting that this effect may also be caused in part by competition between the SMAD2/3 and SMAD1/5/8 pathways for common downstream effectors such as SMAD4 (Fig. 1B). Consistently, transfecting HEK293T cells with CA BMP type 1 receptors (CA-ALK2, CA-ALK3, and CA-ALK6) dramatically suppresses Activin A signaling (Fig. 1G). In contrast to its effects in HEK293T cells, BMP2 has no inhibitory effect on Activin A signaling in LβT2 cells. This suggests that: i) the number of BMP type 1 receptors is even more limiting in LβT2 than in HEK293T cells such that sequestration of 'spare' type 2 receptors by BMP2 does not produce an inhibitory effect and ii) that there is no limiting downstream effector in LβT2 cells (Fig. 2C). The first point, as explained above, is also supported by the equal potencies of AB215 and BMP2 in SMAD1/5/8 signaling in LβT2 cells and the second point is additionally suggested by the fact that transfecting LβT2 cells with CA type 1 BMP receptors (CA-ALK3 and CA-ALK6) does not result in decreased Activin A signaling (Fig. 2F). In accordance with the fact that AB215 is a potent inhibitor of Activin A, AB215 blocks the Activin-induced FSH production in LβT2 cells (Fig. 3C and E).

These data together indicate the role of AB215 both as a potent BMP2-like ligand and as an Activin A antagonist. Our results further reflect the cooperativity between the Activin and BMP pathways in LβT2 cells while clearly indicating the requirement for Activin A signaling in FSHβ induction. This work highlights the importance of cellular context for determining the biological outcome of both native and chimeric TGFβ superfamily ligands. It also demonstrates that AB215 could have medical implications where potent Activin A inhibition coupled with BMP pathway activation is desirable.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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