Normal gonadotropin production and fertility in gonadotrope-specific *Bmpr1a* knockout mice

Xiang Zhou^{1,2}, Ying Wang^{1,2}, Luisina Ongaro^{1,2}, Ulrich Boehm³, Vesa Kaartinen⁴, Yuji Mishina⁴ and Daniel J Bernard^{1,2}

¹Department of Pharmacology and Therapeutics, McGill University, Montreal, Québec, Canada

²Centre for Research in Reproduction and Development, McGill University, Montreal, Québec, Canada

³Department of Pharmacology and Toxicology, University of Saarland School of Medicine, Homburg, Germany

⁴Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA

Correspondence should be addressed to D J Bernard
Email
daniel.bernard@mcgill.ca

Abstract

Pituitary follicle-stimulating hormone (FSH) synthesis is regulated by transforming growth factor β superfamily ligands, most notably the activins and inhibins. Bone morphogenetic proteins (BMPs) also regulate FSH β subunit (*Fshb*) expression in immortalized murine gonadotrope-like L β T2 cells and in primary murine or ovine primary pituitary cultures. BMP2 signals preferentially via the BMP type I receptor, BMPR1A, to stimulate murine *Fshb* transcription *in vitro*. Here, we used a Cre-lox approach to assess BMPR1A's role in FSH synthesis in mice *in vivo*. Gonadotrope-specific *Bmpr1a* knockout animals developed normally and had reproductive organ weights comparable with those of controls. Knockouts were fertile, with normal serum gonadotropins and pituitary gonadotropin subunit mRNA expression. Cre-mediated recombination of the floxed *Bmpr1a* allele was efficient and specific, as indicated by PCR analysis of diverse tissues and isolated gonadotrope cells. Furthermore, BMP2 stimulation of inhibitor of DNA binding 3 expression was impaired in gonadotropes isolated from *Bmpr1a* knockout mice, confirming the loss of functional receptor protein in these cells. Treatment of purified gonadotropes with small-molecule inhibitors of BMPR1A (and the related receptors BMPR1B and ACVR1) suppressed *Fshb* mRNA expression, suggesting that an autocrine BMP-like molecule might regulate FSH synthesis. However, deletion of *Bmpr1a* and *Acvr1* in cultured pituitary cells did not alter *Fshb* expression, indicating that the inhibitors had off-target effects. In sum, BMPs or related ligands acting via BMPR1A or ACVR1 are unlikely to play direct physiological roles in FSH synthesis by murine gonadotrope cells.

Key Words

- ▶ pituitary
- ▶ FSH
- ▶ bone morphogenetic protein
- ▶ activin receptor-like kinase
- ▶ *Cre-lox*

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Introduction

The pituitary glycoprotein follicle-stimulating hormone (FSH) is an essential regulator of ovarian function and testicular development in mammals. FSH synthesis

by pituitary gonadotrope cells is primarily stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus and intrapituitary autocrine/paracrine

factors, the best studied of which are the activins (Bernard *et al.* 2010). Activins are the members of the transforming growth factor β (TGF β) superfamily and signal via complexes of serine/threonine kinase receptors, homolog of *Drosophila* mothers against decapentaplegic (SMAD) proteins, and forkhead box L2 to regulate transcription of the FSH β subunit gene (*Fshb*) (Weiss *et al.* 1995, Suszko *et al.* 2003, Bailey *et al.* 2004, Bernard 2004, Gregory *et al.* 2005, Safwat *et al.* 2005, Suszko *et al.* 2005, Bernard *et al.* 2006, Lamba *et al.* 2006, 2009, 2010, Tran *et al.* 2011, Wang & Bernard 2012, Bernard & Tran 2013). In response to FSH signaling, the gonads synthesize and secrete inhibins, which act in an endocrine fashion to suppress FSH synthesis by competitively binding to activin receptors (Lewis *et al.* 2000, Chapman *et al.* 2002).

Additional members of the TGF β superfamily, the bone morphogenetic proteins (BMPs), have also been implicated in regulating *Fshb* expression both alone and in combination with other factors. For example, BMP6 and BMP7 (at 1 μ g/mL) stimulate ovine *Fshb* promoter-reporter activity in pituitaries of transgenic mice or in transiently transfected murine gonadotrope-like L β T2 cells (Huang *et al.* 2001). By contrast, antisera against BMP7 suppress FSH release from murine, rat, and ovine primary pituitary cultures, suggesting a role for endogenous intrapituitary BMP7 in FSH regulation. BMP15 similarly stimulates ovine *Fshb* promoter activity in L β T2 cells and FSH secretion from rat primary pituitary cell cultures, but at concentrations as low as 10–100 ng/mL (Otsuka & Shimasaki 2002). BMP2 and BMP4 are ten-fold more potent in stimulating murine *Fshb* promoter-reporter activity than BMP6 or BMP7 in L β T2 cells (Lee *et al.* 2007). Moreover, BMP2 and activins synergistically stimulate murine, porcine, and ovine *Fshb* promoter-reporter activities as well as endogenous murine *Fshb* mRNA expression in this cell line. Similarly, BMP4 potentiates activin A and GnRH induction of *Fshb* mRNA expression and FSH release in L β T2 cells (Nicol *et al.* 2008), whereas BMP6 synergistically stimulates murine *Fshb* promoter-reporter activity with GnRH (Takeda *et al.* 2012). In contrast to these results, exogenous BMP4 and BMP6 suppress FSH secretion and *Fshb* mRNA expression in primary pituitary cultures from ewes and also counteract the stimulatory effects of activin A in this system (Faure *et al.* 2005, Young *et al.* 2008). Collectively, these data suggest that BMPs can regulate FSH at the level of the pituitary gland, though their effects may be ligand-, context-, and species-specific.

In L β T2 cells, BMP2 signals via the type I receptor, BMPR1A (also known as activin receptor-like kinase 3 or ALK3) to regulate murine *Fshb* transcription (Ho & Bernard 2009). Though a second type I receptor, BMPR1B (ALK6), is also present in these cells (Lee *et al.* 2007, Nicol *et al.* 2008), it is expressed at low levels and BMP2 signaling is intact when this receptor is knocked down using short interfering RNAs (Ho & Bernard 2009). Similarly, in sheep, BMPR1A, but not BMPR1B, is expressed in gonadotrope cells (Faure *et al.* 2005), suggesting that BMP2 and BMP4 likely signal via BMPR1A in these animals as well. Here, we tested the hypothesis that signaling via BMPR1A is required for FSH synthesis *in vivo* by selectively ablating the receptor in murine gonadotropes using a Cre-lox approach.

Materials and methods

Reagents

Human recombinant (rh-) BMP2 (355-BM) and activin A (338-AC) were from R&D Systems. RQ1 RNase-Free DNase (M6101), random primers (C1181), MMLV-reverse transcriptase (M1701), and RNasin (N2511) were from Promega. SB431542 (S4317), pancreatin (P3292), and collagenase (Type I-C0130) were from Sigma. Media 199 (M199; 31100-035), Hanks' Balanced Salt Solution (HBSS) without calcium/magnesium (14170-112), TRIzol Reagent, and SYBRgreen Supermix for quantitative PCR (qPCR) were from Invitrogen. EvaGreen 2X qPCR MasterMix-S was from Applied Biological Materials, Inc. (Richmond, BC, Canada). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Gentamycin (450-135-XL), 100X antibiotic-antimycotic (450-115-EL), and deoxynucleotide triphosphates were from Wisent (St-Bruno, QC, Canada). LDN-193189 hydrochloride (1509) was purchased from Axon Medchem (Reston, VA, USA), and compound C (171260) was from Calbiochem (EMD Chemicals, Inc., Darmstadt, Germany).

Animals

The mouse strains used here have been described previously: *Bmpr1a*^{+/-} (Mishina *et al.* 1995), *Bmpr1a*^{fl/fl} (Mishina *et al.* 2002), *Acvr1*^{fl/fl} (Dudas *et al.* 2004), and GnRH-receptor-IRES-Cre (GRIC) (Wen *et al.* 2008). In the latter model, Cre recombinase is expressed as part of a bicistronic mRNA with the endogenous *Gnrhr* mRNA. *Bmpr1a*^{+/-} males were crossed with *Gnrhr*^{GRIC/GRIC} females.

Resulting *Bmpr1a*^{+/-};*Gnrhr*^{GRIC/+} females were then crossed with *Bmpr1a*^{fl/fl} males to generate control (*Bmpr1a*^{fl/+};*Gnrhr*^{GRIC/+}) and experimental animals (*Bmpr1a*^{fl/-};*Gnrhr*^{GRIC/+}). The GRIC allele is active in the male germ line (Wen *et al.* 2010); therefore, to avoid global recombination of the floxed allele, GRIC was always introduced from the female parent. The *Bmpr1a*^{+/-};*Gnrhr*^{GRIC/+} × *Bmpr1a*^{fl/fl} cross generated animals of four genotypes at the expected frequencies (1:1:1:1). For genetic labeling of gonadotropes, *Gnrhr*^{GRIC/GRIC} mice were crossed with *Rosa26-loxSTOPlox-EYFP* (hereafter R26-YFP) reporter mice (Srinivas *et al.* 2001) acquired from Jackson Laboratories. To label gonadotropes in control and experimental mice, we crossed *Bmpr1a*^{+/-};*Gnrhr*^{GRIC/GRIC} females with *Bmpr1a*^{fl/fl};R26-YFP/R26-YFP males. *Acvr1*^{fl/fl} and *Bmpr1a*^{fl/fl} mice were crossed to generate *Acvr1*^{fl/+}; *Bmpr1a*^{fl/+} and eventually *Acvr1*^{fl/fl}; *Bmpr1a*^{fl/fl} mice for *in vitro* recombination experiments. All animals were housed on a 12h light:12h darkness cycle and were given access to food and water *ad libitum*. All mouse work was conducted in accordance with federal and institutional guidelines and with the approval of the McGill Animal Care and Use Committee (animal use protocol #5204).

DNA extraction and genotyping

Genomic DNA was extracted from tail biopsies (~0.5 cm) using 0.5 mL of lysis buffer (100 mmol/L Tris-HCl (pH 8.5), 5 mmol/L EDTA (pH 8.0), 200 mmol/L NaCl, 0.2% (v/v) SDS, and 100 µg/mL proteinase K). Tails were incubated at 55°C overnight in a water bath. Samples were then vortexed and centrifuged at 14,600g for 10 min. The supernatant was collected and mixed by inversion with 0.5 mL isopropanol. Precipitated DNA was collected with a micropipette tip and dissolved in 40 µL of 10 mmol/L Tris (pH 8.0). For comparison of recombination across different tissues, DNA was extracted from approximately 5 mg of the indicated tissues using the Gentra Puregene Blood Kit (Qiagen) following the manufacturer's instructions. Wild-type, null, floxed, and recombined *Bmpr1a* alleles were detected by PCR using the protocols described in (Mishina *et al.* 1995, 2002). The GRIC allele was detected using the primer set indicated in Table 1.

Hormone assays

Serum luteinizing hormone (LH) and FSH were measured by multiplex ELISA at the Ligand Assay and Analysis Core (LAAC) of the Center for Research in Reproduction at the

University of Virginia (Charlottesville, VA, USA). Both hormones were measured in singlet from 10 µL serum. The reportable ranges for LH and FSH were 0.24–30.0 and 2.40–300.0 ng/mL, respectively.

Fluorescence-activated cell sorting of genetically labeled gonadotropes

Pituitaries from mice with yellow fluorescent protein (YFP)-labeled gonadotropes were enzymatically dispersed as described in (Ho *et al.* 2011) and single-cell suspensions were prepared in PBS. Cells were then passed through a 70 µm nozzle at 70 psi into a Becton Dickinson FACS Aria Sorter in the McGill University Flow Cytometry Core Facility. Sorting was performed using FACSDiva software (v. 6.0). Gating was established on a forward and side scatter plot (for relative cell size and granularity, respectively) to exclude debris and cell clusters. A control without YFP-labeled cells was run to establish a negative

Table 1 Genotyping and quantitative RT-PCR primers.

Genotyping

Cre

Fwd: GGACATGTTTCAGGGATCGCCAGGC
Rev: GCATAACCACTGAAACAGCATTGCTG

Acvr1

Fwd: CCCCCATTGAAGGTTTAGAGAGAG
Rev: CTAAGAGCCATGACAGAGGTTG

Bmpr1a

Fwd1: CTCTGAATTTCTAGTCCACATCTGC
Fwd2: AGACTGCCTTGGGAAAAGCGC
Rev: GGACTATGGACACACAATGGC

Quantitative RT-PCR

Acvr1

Fwd: GCTGCATAGCAGATTTGGGC
Rev: CACTTCCGGAGCCATGTAGC

Bmpr1a

Fwd: CATCAGATTACTGGGAGCCTG
Rev: GGTATCCTCTGGTGCTAAAGTC

Bmpr1b

Fwd: GTCTCAGAGCTCGGGAAGTG
Rev: ATAGCGGCCTTTTCCAATCT

Fshb

Fwd: GTGCGGGCTACTGCTACACT
Rev: CAGGCAATCTTACGGTCTCG

Id1

Fwd: GGTACTTGGTCTGTCGGAGC
Rev: GCAGGTCCCTGATGTAGTCG

Id3

Fwd: TTAGCCAGGTGGAAATCCTG
Rev: TCAGTGGCAAAAGCTCCTCT

Lhb

Fwd: ACTGTGCCGGCCTGTCAACG
Rev: AGCAGCCGGCAGTACTCGGA

Rpl19

Fwd: CGGGAATCCAAGAAGATTGA
Rev: TTCAGCTTGTTGGATGTGCTC

Figure 1

(A) PCR analysis of genomic DNA from the indicated tissues in cKO male and female mice. The presence of a band indicates recombination of the 'floxed' *Bmpr1a* allele. (B) PCR analysis of genomic DNA from purified gonadotrope (YFP⁺, right) and non-gonadotrope (YFP⁻, left) cells from pituitaries of adult male control and cKO mice. Top: Primer set that detects the floxed (top band) or wild-type/null (bottom band) *Bmpr1a* alleles. Bottom: Primer set that detects the recombined *Bmpr1a* allele. (C) Gonadotropes (YFP⁺) purified from control and cKO mice were plated in primary culture and then treated with vehicle or 25 ng/mL BMP2 for 24 h. *Id3* mRNA expression was measured by quantitative RT-PCR. Data are means ($\pm$ S.E.M.) of five independent experiments. Data from male and female cultures did not differ and were combined for analysis. ** indicates different from all other groups as determined by two-way ANOVA of log transformed data ($P < 0.01$).

baseline profile using a single-parameter histogram. The pituitary cells were then run and gated to sort YFP⁺ and YFP⁻ cells. YFP was excited with a 488 nm argon ion laser and detected with a 530/30 band-pass filter and an LP 502 dichroic mirror. Depending on the preparation, we obtained roughly $0.8\text{--}15 \times 10^3$ YFP⁺ and $4.6\text{--}18 \times 10^4$ YFP⁻ cells per pituitary.

Gonadotrope cell culture

Purified gonadotropes were plated at a density of $4\text{--}5 \times 10^4$ cells per well in 96-well plates in Medium 199 with 10% fetal bovine serum (FBS), 1X antibiotic-antimycotic, and 120 $\mu\text{g/mL}$ gentamicin. Cells were incubated at 37°C/5% CO₂ for at least 16 h before treatment. Cells were treated in medium M199 plus 2% (v/v) FBS overnight with the indicated ligands and/or inhibitors. RNA and DNA were then collected with AllPrep DNA/RNA mini kits (cat. 80204; Qiagen) following the manufacturer's instructions. DNA was analyzed as described previously. RNA was analyzed by quantitative RT-PCR (discussed later).

Primary pituitary cultures

Primary cultures were performed as described previously (Fortin *et al.* 2013). Briefly, pituitaries were

collected from 10-week-old *Bmpr1a*^{fl/fl};*Acvr1*^{fl/fl} male and female mice in M199 medium supplemented with 10% (v/v) FBS. Pituitaries were washed 3 times in HBSS with 150 $\mu\text{mol/L}$ of CaCl₂, cut several times with a scalpel, and digested in collagenase (1.5 mg/mL) (Sigma #C-0130; diluted in HBSS with 30 mg/mL BSA, pH 7.4, 40 $\mu\text{L/pituitary}$) at 37°C for 2 h. The suspension was then washed with 5 mL calcium-free HBSS, centrifuged for 5 min at 1000g, and resuspended in pancreatin solution (Sigma P3292; 4.5 mg/mL in calcium-free HBSS; 40 $\mu\text{L/pituitary}$). Pancreatin digestion was performed in a 37°C water bath with manual agitation for 8–10 min. Finally, the cell suspension was washed 3 times in 5 mL M199 media containing 10% FBS and cells were seeded at density of $5 \times 10^5/\text{well}$ in 48-well plates.

Adenoviral transduction and treatment of primary pituitary cultures

Primary cultures were transduced with adenoviruses as described previously (Fortin *et al.* 2014). Pituitary cultures were prepared as described previously. After 24 h, viral transductions were performed using adenoviruses that express enhanced green fluorescent protein (eGFP) or Cre-IRES-eGFP (Vector Development Laboratory, Baylor College of Medicine, Houston, TX, USA) at a multiplicity of infection of 60 in M199 medium

Figure 2

Female and male control and cKO mice were paired with wild-type C57BL/6 mice for a period of 6 months ($N=6-8$ per sex per genotype). (A) Number of days since pairing until delivery of the first litter, (B) average number of litters per pair, (C) average number of pups per litter, and (D) average number of days between litters. Data are means ($\pm$ S.D.). (Data were analyzed with unpaired t -tests; NS, not significantly different ($P>0.05$)).

containing 10% (v/v) FBS. The following day, virus-containing medium was removed and replaced with medium containing 2% (v/v) FBS with 25 ng/mL activin A, 25 ng/mL BMP2, 1 μ mol/L SB431542 or 1 μ mol/L LDN-193189, or DMSO as vehicle. All treatments were performed for 24h in duplicate, and the experiment was repeated 5–7 times. Cells were harvested, and RNA and DNA were extracted using the Allprep DNA/RNA kit (Qiagen). RNA was eluted in 23 μ L RNase-free water and

reverse transcribed. The resulting cDNA was analyzed by qPCR.

Quantitative RT-PCR

Fshb and *Lhb* mRNA levels (normalized to *Rpl19*) in whole pituitary RNA were measured using the relative standard curve method with L β T2 cell RNA as standard, as described previously (Lamba *et al.* 2009). *Fshb*, *Lhb*, *Id3*, *Bmpr1a*, *Bmpr1b*, *Acvr1a*, and *Id* mRNA levels (normalized to *Rpl19*) in isolated gonadotropes or mixed pituitary cultures were measured using the $2^{-\Delta\Delta C_t}$ method as described in (Ho & Bernard 2010). Primer sequences are indicated in Table 1.

Statistics

Data from control and experimental mice and/or cell cultures were compared with t -tests or analyses of variance as indicated using Systat (Systat Software Inc, San Jose, CA, USA). *Post hoc* pair-wise comparisons were made with Bonferroni corrections. Data were log transformed when variances were unequal. Significance was assessed relative to $P<0.05$.

Results

Gonadotrope-specific *Bmpr1a* knockout mice

We generated gonadotrope-specific *Bmpr1a* knockout mice by crossing 'floxed' *Bmpr1a* animals with mice expressing Cre recombinase from the endogenous *Gnrhr* locus (so-called GRIC mice, see the 'Materials and methods' section). Because *Bmpr1a*^{+/-} mice are viable and fertile, we elected to cross in a single null allele such that only one floxed *Bmpr1a* allele required recombination in our model. As a result, the experimental mice (hereafter

Table 2 Body and reproductive organ weights.

Sex	Genotype	N	Body weight (g)		Ovarian weight (g)*		Uterine weight (g)	
F	Control	10	17.65 $\pm$ 0.26	$P>0.1$	0.0084 $\pm$ 0.001	$P>0.1$	0.110 $\pm$ 0.013	$P>0.1$
F	cKO	10	18.27 $\pm$ 0.45		0.011 $\pm$ 0.001		0.092 $\pm$ 0.013	
Sex	Genotype	N	Body weight (g)		Testicular weight (g)*			
M	Control	11	21.36 $\pm$ 0.7	$P>0.1$	0.168 $\pm$ 0.006	$P<0.01$		
M	cKO	14	22.68 $\pm$ 0.8		0.206 $\pm$ 0.008			

Data are means $\pm$ S.E.M. *Paired organ weights. Data were analyzed with unpaired t -tests. P values are presented for each comparison. F, female; M, male.

Figure 3

Serum and pituitaries were collected from control and cKO mice at 6 weeks of age. (A) LH and (B) FSH were measured by ELISA. RNA was extracted from whole pituitaries. (C) *Lhb* and (D) *Fshb* mRNA levels were assessed by quantitative RT-PCR. Hormone data reflect means ($\pm$ S.E.M.) of $N=8$ per sex per genotype. RNA data are means ($\pm$ S.E.M.) of $N=5$ per sex per genotype. Data were analyzed with unpaired *t*-tests; NS, not significantly different ($P>0.05$).

conditional knockouts or cKOs) had the following genotype: *Bmpr1a*^{fl/-};*Gnrhr*^{GRIC/+}. Control littermates from the *Bmpr1a*^{+/-};*Gnrhr*^{GRIC/+} $\times$ *Bmpr1a*^{fl/fl} cross used to generate cKO mice had the following genotype: *Bmpr1a*^{fl/+};*Gnrhr*^{GRIC/+}. These animals allowed us to control for potential effects of the GRIC allele. Recombination of a single floxed allele in gonadotropes of these animals was not a concern because, as mentioned previously, *Bmpr1a*^{+/-} mice are fertile. Mice of the two other genotypes derived from this cross (*Bmpr1a*^{fl/+};*Gnrhr*^{+/+} or *Bmpr1a*^{fl/-};*Gnrhr*^{+/+}) were not analyzed. The GRIC allele is active in pituitary gonadotropes and in male germ cells. Therefore, as anticipated, we observed the recombination of the floxed *Bmpr1a* allele in pituitaries, testes, and epididymides, but not in other tissues, including ovaries and uteri of adult cKOs (Fig. 1A).

To further demonstrate the efficacy of recombination, we genetically labeled and purified gonadotropes from control and cKO mice. Here, an enhanced YFP (eYFP) reporter allele, which is activated by Cre recombinase-mediated removal of a transcriptional stop cassette, was introduced into the genetic background of the control

and cKO strains. Their pituitaries were extracted and enzymatically dissociated. eYFP-labeled (YFP⁺) cells were then separated from eYFP-negative (YFP⁻) cells by FACS. DNA was extracted from the purified cell populations and analyzed by PCR. The data confirm the efficient recombination of the floxed *Bmpr1a* allele in YFP⁺, but not YFP⁻ cells in males of both genotypes (Fig. 1B; recall that both control and cKO mice harbor a single floxed *Bmpr1a* allele; therefore, recombination is predicted in both genotypes). Similar results were obtained in females (data not shown).

We previously showed that BMP2 signals via BMPR1A to regulate *Id3* expression in L β T2 cells and in primary pituitary cell cultures (Ho *et al.* 2011). Therefore, to confirm functional deletion of BMPR1A, we treated purified gonadotropes from control and cKO mice in primary culture with 25 ng/mL BMP2 for 24 h. BMP2-stimulated *Id3* mRNA expression was significantly reduced in cKOs relative to control gonadotropes (Fig. 1C).

Fertility is normal in *Bmpr1a* cKO mice

Eight- to ten-week-old control and cKO males and females were paired with wild-type C57BL/6 (Charles River) opposite sex partners for a period of 6 months to assess their fertility. As shown in Fig. 2, there were no significant differences between genotypes in the number of days from pairing to delivery of the first litter, the average numbers of litters per animal, the average number of pups per litter, or the inter-litter interval.

Reproductive tissues and hormone levels are normal in *Bmpr1a* cKO mice

We next measured reproductive organ weights (Table 2), serum gonadotropin levels (Fig. 3A and B), and pituitary *Lhb* and *Fshb* mRNA levels (Fig. 3C and D) in postpubertal (6-week-old) control and cKO mice of both sexes. Again, we did not observe any differences between genotypes in any of these parameters, with the exception of testicular weight, which was slightly elevated in cKO mice.

BMP2 does not stimulate *Fshb* mRNA expression in purified gonadotropes

Previous analyses of BMP2-regulated *Fshb* expression were conducted almost entirely in the L β T2 cell model

Figure 4

Gonadotropes were purified from wild-type (GRIC-YFP) mice and plated in primary culture. Cells were treated with 25 ng/mL activin A or BMP2 overnight. RNA was extracted and (A) *Fshb* or (B) *Id3* mRNA levels were measured by quantitative RT-PCR. The data reflect the means ($\pm$ S.E.M.) of five (*Id3*) or six (*Fshb*) independent experiments (two or three cultures were from males, two from females, and one from mixed sex cultures). ***indicates different from control as determined by one-way ANOVA of log transformed data ($P < 0.001$). (C) Cells were treated with 1 μ mol/L SB431542 or LDN-193189 (or DMSO vehicle) overnight. RNA was extracted and *Fshb* mRNA levels were measured by quantitative RT-PCR. Data are the means ($\pm$ S.E.M.) of three (SB) or five (LDN) independent experiments (three cultures were from females and two from males). ***indicates different from control as determined by one-way ANOVA ($P < 0.001$).

(Lee *et al.* 2007, Ho & Bernard 2009, 2010, Ho *et al.* 2011). We therefore asked whether BMP2 similarly regulates *Fshb* expression in isolated murine gonadotropes of wild-type mice. Whereas activin A robustly stimulated *Fshb* mRNA in these cells, BMP2 had no effect (Fig. 4A). By contrast, BMP2 stimulated and activin A inhibited *Id3* mRNA expression in these cultures (Fig. 4B). Thus, BMP2 was active in primary gonadotropes but did not regulate the *Fshb* gene therein.

Inhibitors of BMP type I receptors suppress *Fshb* expression in primary gonadotropes

Activin B is generally regarded as the primary autocrine/paracrine stimulator of *Fshb* transcription (Corrigan *et al.* 1991, Sallon *et al.* 2010). Consistent with this idea, treatment of isolated gonadotropes from wild-type mice with a small-molecule inhibitor (SB431542) (Inman *et al.* 2002) of the known activin B type I receptors (ACVR1B and ACVR1C) (Tsuchida *et al.* 2004, Bernard *et al.* 2006) dramatically reduced *Fshb* expression (Fig. 4C). To determine whether autocrine/paracrine BMP-like molecules (other than BMP2) might also play a role in *Fshb* expression, we also treated gonadotropes with LDN-193189 (Cuny *et al.* 2008). This molecule blocks the activities of ACVR1, BMPR1A, and BMPR1B, the three best-characterized BMP type I receptors (for convenience, these receptors will be referred to as ALK2/3/6). LDN-193189 also suppressed *Fshb* mRNA levels (Fig. 4C). We obtained similar results with a second ALK2/3/6 inhibitor, compound C (also known as dorsomorphin; Yu *et al.* 2008) (data not shown).

Fshb production by primary pituitary cultures is ACVR1/BMPR1A-independent

Though these data suggested a role for endogenous BMP-like molecules (other than BMP2) in FSH synthesis, LDN-193189 and compound C are known to antagonize other kinases in cells (Vogt *et al.* 2011). To assess potential 'off-target' effects, we examined the inhibitor activity in pituitary cells expressing or lacking BMPR1A and ACVR1. It should be noted that BMPR1B/ALK6 is expressed at very low levels (if at all; Yi *et al.* 2001) in the pituitary and in these cultures. Though we did not perform absolute quantification, the Ct values for *Bmpr1b* were 3–5 cycles higher than for *Acvr1* or *Bmpr1a*, a difference of 8- to 32-fold. Similarly, a recent RNA-seq analysis indicates that *Bmpr1a* and *Acvr1* mRNA levels are ~32- and ~16-fold greater than *Bmpr1b* in purified murine gonadotropes (Qiao *et al.* 2016). We prepared pituitary cultures from mice harboring floxed alleles for both BMPR1A (ALK3) and ACVR1 (ALK2) (*Bmpr1a^{fl/fl}*; *Acvr1^{fl/fl}*). In these experiments, the cultures were prepared from whole pituitaries rather than from purified gonadotropes (it was not feasible to perform these experiments in purified gonadotropes). Half of the cells were infected with a control adenovirus expressing GFP. The other half was infected with a Cre-expressing adenovirus to recombine the floxed alleles. As shown in Fig. 5A and B, *Acvr1* and *Bmpr1a* mRNA levels were significantly depleted in cells transduced with the Cre adenovirus. There was no compensatory increase in *Bmpr1b* mRNA expression (Fig. 5C). Stimulation of *Id1* expression by BMP2 (Hollnagel *et al.* 1999) was significantly impaired in Cre adenovirus-treated cells,

Figure 5

Primary pituitary cultures were prepared from *Bmpr1a^{fl/fl};Acvr1^{fl/fl}* mice. Cells were then transduced with GFP or Cre-encoding adenoviruses before being treated 24 h later with 25 ng/mL activin A, 25 ng/mL BMP2, 1 µmol/L SB431542, or 1 µmol/L LDN-193189 in 2% (v/v) serum-containing medium. Twenty-four hours later, RNA was collected and *Acvr1a* (A), *Bmpr1a* (B), *Bmpr1b* (C), *Id1* (D), *Fshb* (E), or *Lhb* (F) mRNA levels were measured by quantitative RT-PCR. The data presented reflect the means ($\pm$ s.e.m.) of four to seven independent experiments. Cultures for females and males were run separately, but then combined for analyses when no sex differences were noted. All data were analyzed by two-way ANOVA (ligand $\times$ virus). *Post hoc* pair-wise comparisons (Bonferroni) were made of interactions (panels A, B, and D) or significant main effects (panel E). *** $P < 0.001$ for GFP vs Cre (panels A and B), compared with all other treatments (panel D), or compared with no ligand treatment.

demonstrating the loss of functional type I receptor proteins (Fig. 5D). The loss of the receptors, however, did not alter (reduce) basal *Fshb* mRNA expression (Fig. 5E). Thus, endogenous TGF β superfamily ligands regulate *Fshb* expression independently of these receptors. Importantly, both LDN-193189 and compound C impaired *Fshb* mRNA expression in mixed pituitary cultures (though to a lesser extent than in purified gonadotropes; compare Fig. 5E with Fig. 4C) whether the *Bmpr1a* and *Acvr1* alleles were recombined or not. Therefore, these inhibitors appear to suppress *Fshb* expression in a BMPR1A/ACVR1-independent fashion. Again, activin A, but not BMP2, stimulated *Fshb* mRNA levels in these cultures (Fig. 5E). None of the treatments affected *Lhb* expression (Fig. 5F).

Discussion

The data presented here indicate that BMPR1A is dispensable for normal gonadotropin synthesis and fertility in mice

in vivo. Though these observations do not definitively rule out a role for BMP signaling in FSH regulation, they do suggest that any biologically relevant BMP-like ligands signal via an alternative type I receptor in the absence of BMPR1A (i.e., there is some form of compensation) and/or that these ligands actually prefer a receptor other than BMPR1A.

We focused on BMPR1A for *in vivo* analyses based on earlier results with BMP2 and BMP4 in L β T2 cells (Lee *et al.* 2007, Ho & Bernard 2009, 2010, Ho *et al.* 2011) and with BMP4 in ovine pituitary cultures (Faure *et al.* 2005, Young *et al.* 2008). Both of these ligands preferentially signal via BMPR1A and BMPR1B compared with the other type I receptors in the family (ten Dijke *et al.* 1994, Liu *et al.* 1995). BMPR1B is expressed at low levels, if at all, in gonadotropes (Lee *et al.* 2007, Nicol *et al.* 2008, Qiao *et al.* 2016) and BMP2 preferentially signals via BMPR1A in L β T2 cells to regulate both *Fshb* and *Id3* transcription (Ho & Bernard 2009, Ho *et al.* 2011). The latter data were collected in the context of transient transfection experiments, which may have precluded compensatory

mechanisms from developing. In the *in vivo* knockout model presented here, Cre expression occurs as early as embryonic day 12.75 (Wen *et al.* 2010), and we only examined mice in adulthood. As a result, it is possible that another receptor(s) could have compensated for the absence of BMPR1A during this protracted time frame. That said, *Bmpr1b* mRNA levels are low in isolated murine gonadotropes under normal conditions (Qiao *et al.* 2016) and do not increase in *Bmpr1a* cKO mice (data not shown).

BMP2 and BMP4 can also signal via the type I receptor ACVR1 (ALK2) (Liu *et al.* 1995, Macias-Silva *et al.* 1998), which is expressed in murine gonadotropes (Qiao *et al.* 2016). Nonetheless, BMP2 signaling in isolated gonadotropes from *Bmpr1a* cKO mice was greatly impaired (Fig. 1C), arguably ruling out a compensatory role for ACVR1 or another type I receptor, at least for this particular BMP ligand.

To determine whether or not ACVR1 can compensate for the loss of BMPR1A or is perhaps the preferred type I receptor for the biologically relevant BMP ligands *in vivo*, one could conditionally ablate the receptor (Kaartinen & Nagy 2001, Dudas *et al.* 2004) in gonadotropes either alone or in combination with BMPR1A (Yoon *et al.* 2005, Orvis *et al.* 2008, Edson *et al.* 2010). We have not systematically analyzed such mice to the extent that we have with *Bmpr1a* cKOs. However, we did generate a small number of *Gnrhr^{GRIC/+}; Bmpr1a^{fl/fl}; Acvr1^{fl/fl}* mice, which lack both receptors in gonadotropes. Thus far, double knockout females produce litters of normal size. This would suggest that FSH (and LH) synthesis is unimpaired, though we have not assessed this directly. Indeed, in light of these preliminary observations and the results of the *in vitro* recombination experiments presented in Fig. 5, a thorough analysis of the double knockouts appears to be unjustified.

We pursued BMP2 and BMPR1A (by extension) because BMP2 and BMP4 were ten-fold more potent than BMP6 or BMP7 in stimulating *Fshb* transcription in L β T2 cells (Lee *et al.* 2007). As BMP2 and BMP4 are expressed at relatively low levels in gonadotropes (Lee *et al.* 2007, Qiao *et al.* 2016), we argued that they would most likely regulate *Fshb* in a paracrine fashion. However, in light of our observations here with conditional *Bmpr1a* knockout mice and previous data implicating endogenous BMP7 in FSH regulation (Huang *et al.* 2001), it is fair to question whether a singular focus on BMP2 and its canonical receptor BMPR1A might have been limiting. That is, perhaps autocrine BMP7 is the primary BMP ligand regulating FSH *in vivo*. Indeed, *Bmp7* is among the most highly expressed BMP ligands in isolated gonadotropes (Qiao *et al.* 2016). However, ACVR1 is the preferred

BMP7 type I receptor (Macias-Silva *et al.* 1998), and the recombination experiments in Fig. 5 rule out a role for an endogenous ligand that signals via this receptor in *Fshb* synthesis, at least in primary culture.

In sum, though *in vitro* analyses in the gonadotrope-like L β T2 cell line suggested a role for BMP2 as a regulator of *Fshb* transcription both alone and in synergy with activins, the data presented here demonstrate that the ligand fails to stimulate *Fshb* expression in isolated primary gonadotropes and that its primary type I receptor, BMPR1A, is not required for normal FSH production or fertility in mice. Small-molecule BMPR1A, BMPR1B, and ACVR1 inhibitors suppressed *Fshb* expression in primary cells, suggesting a role for endogenous BMPs or related ligands in FSH synthesis. However, it now appears that the inhibitors' effects were likely independent of BMP signaling as the genetic deletion of BMPR1A and ACVR1 together did not alter *Fshb* production in the same cells. In light of these data, we suggest that studies on the direct regulation of FSH synthesis by TGF β superfamily ligands should focus on activins and related proteins that signal via ACVR1B (ALK4), TGFBR1 (ALK5), and/or ACVR1C (ALK7).

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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Author contribution statement

X Z performed all of the *in vivo* experiments; prepared, treated, and analyzed the pituitary cultures; and edited the manuscript. Y W performed several of the quantitative RT-PCR analyses and edited the manuscript. L O wrote sections of the manuscript and edited the final version. U B, V K, and Y M produced the floxed and Cre strains, provided advice regarding experimental design, and edited the manuscript. D J B designed the experiments, analyzed the data, generated the final figures and tables, wrote the original draft of the manuscript, and edited and formatted the final version of the manuscript.

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