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Normal gonadotropin production and fertility in gonadotrope-specific BMPR1A knockout mice

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Abstract

Pituitary FSH synthesis is regulated by TGFβ superfamily ligands; most notably the activins and inhibins. Bone morphogenetic proteins (BMPs) also regulate FSHβ subunit (*Fshb*) expression in immortalized murine gonadotrope-like LβT2 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 to 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.

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Declaration of interest

The authors have no conflicts of interest to disclose.

Author contributions

XZ performed all of the *in vivo* experiments; prepared, treated, and analyzed the pituitary cultures; and edited the manuscript. YW performed several of the RT-qPCR analyses and edited the manuscript. LO wrote sections of the manuscript and edited the final version. UB, VK, and YM produced the floxed and Cre strains, provided advice regarding experimental design, and edited the manuscript. DJB designed the experiments, analyzed the data, generated the final figures and tables, wrote the original draft of manuscript, and edited and formatted the final version of the manuscript.

Keywords

Pituitary; FSH; bone morphogenetic protein; activin receptor-like kinase; Cre-lox

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 intra-pituitary autocrine/paracrine factors, the best studied of which are the activins (Bernard *et al.*, 2010). Activins are members of the transforming growth factor β (TGFβ) 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, Lamba *et al.*, 2009, Lamba *et al.*, 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 endocrine fashion to suppress FSH synthesis by competitively binding to activin receptors (Lewis *et al.*, 2000, Chapman *et al.*, 2002).

Additional members of the TGFβ 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 μg/ml) stimulate ovine *Fshb* promoter-reporter activity in pituitaries of transgenic mice or in transiently transfected murine gonadotrope-like LβT2 cells (Huang *et al.*, 2001). In contrast, antisera against BMP7 suppress FSH release from murine, rat, and ovine primary pituitary cultures, suggesting a role for endogenous intra-pituitary BMP7 in FSH regulation. BMP15 similarly stimulates ovine *Fshb* promoter activity in LβT2 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 LβT2 cells (Lee *et al.*, 2007). Moreover, BMP2 and activins synergistically stimulate murine, porcine, and ovine *Fshb* promoter-reporters 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 LβT2 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 LβT2 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 (siRNAs) (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 (Minneapolis, MN, USA). RQ1 RNase-Free DNase (M6101), random primers (C1181), MMLV-reverse transcriptase (M1701) and RNasin (N2511) were from Promega (Madison, WI, USA). SB431542 (S4317), pancreatin (P3292), and collagenase (Type I-C0130) were from Sigma (St. Louis, MO, USA). Media 199 (M199; 31100-035), Hanks' Balanced Salt Solution (HBSS) without calcium/magnesium (14170-112), TRIzol Reagent, and SYBRgreen Supermix for qPCR were from Invitrogen (Burlington, ON, Canada). EvaGreen 2X qPCR MasterMix-S was from Applied Biological Materials Inc. (ABM, Richmond, BC, Canada). Oligonucleotides were purchased from IDT (Coralville, IA, USA). Gentamycin (450-135-XL), 100X antibiotic-antimycotic (450-115-EL) and deoxynucleotide triphosphates (dNTPs) were from Wisent (St-Bruno, Quebec, 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 germline (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 labs. 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 12L:12D light cycle and were given *ad libitum* access to food and water. 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^{-1} Tris HCl (pH 8.5), 5 mmol l^{-1} EDTA (pH 8.0), 200 mmol l^{-1} 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 12,000 rpm 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^{-1}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 following the manufacturer's instructions (Qiagen). Wild-type, null, floxed, and recombined *Bmpr1a* alleles were detected by PCR using the protocols described in (Mishina *et al.*, 1995, Mishina *et al.*, 2002). The GRIC allele was detected using the primer set indicated in Table 1.

Hormone assays

Serum 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. Both hormones were measured in singlet from 10 μl serum. The reportable ranges for LH and FSH were 0.24–30.0 ng/ml and 2.40–300.0 ng/ml, respectively.

Fluorescence activated cell sorting of genetically-labeled gonadotropes

Pituitaries from mice with YFP-labeled gonadotropes were enzymatically dispersed as described in (Ho *et al.*, 2011) and single cell suspensions prepared in PBS. Cells were then passed through a 70 μm nozzle at 70 psi into a Becton Dickinson FACSAria 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 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 a LP 502 dichroic mirror. Depending on the preparation, we obtained roughly 0.8–15 × 10^3 YFP+ and 4.6–18 × 10^4 YFP− cells per pituitary.

Gonadotrope cell culture

Purified gonadotropes were plated at a density of 4–5 ×10^4 cells per well in 96-well plates in Medium 199 with 10% fetal bovine serum (FBS), 1X antibiotic-antimycotic, and 120 μg/ml gentamicin. Cells were incubated at 37°C/5% CO_2 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 QIAGEN AllPrep DNA/RNA mini kits (cat. 80204) following the manufacturer’s instructions. DNA was analyzed as described above. RNA was analyzed by quantitative RT-PCR (below).

Primary pituitary cultures

Primary cultures were performed as previously described (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) fetal bovine serum (FBS). Pituitaries were washed three times in Hank's Balanced Salt Solution (HBSS) with 150 μmol l^{-1} of $CaCl_2$, 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 μl/pituitary) at 37°C for 2 hours. The suspension was then washed with 5 ml calcium-free HBSS, centrifuged for 5 min at 1000 × g, and resuspended in pancreatin solution (Sigma P3292; 4.5 mg/ml in calcium-free HBSS; 40 μl/pituitary). Pancreatin digestion was performed in a 37°C water bath with manual agitation for 8 to 10 min. Finally, the cell suspension was washed three times in 5 ml M199 media containing 10% FBS and cells were seeded at density of 5×10^5/well in 48-well plates.

Adenoviral transduction and treatment of primary pituitary cultures

Primary cultures were transduced with adenoviruses as previously described (Fortin *et al.*, 2014). Pituitary cultures were prepared as described above. After 24 hours, viral transductions were performed using adenoviruses that express enhanced green fluorescent protein (eGFP) or Cre-IRES-eGFP (Baylor College of Medicine Vector Development Laboratory, Houston, TX. USA) at a multiplicity of infection of 60 in M199 medium 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 μmol l^{-1} SB431542 or 1μmol l^{-1} LDN-193189 or DMSO as vehicle. All treatments were performed for 24 hours in duplicate and the experiment was repeated 5–7 times. Cells were harvested and RNA and DNA were extracted using the Qiagen Allprep DNA/RNA kit. RNA was eluted in 23 μl 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 LβT2 cell RNA as standard, as previously described (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 $2^{-\Delta\Delta Ct}$ 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. Post-hoc pair-wise comparisons were made with Bonferroni corrections. Data were log transformed when variance 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 *Materials and Methods*). 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 conditional knockouts or cKO) had the following genotype: $Bmpr1a^{fl/-}$;$Gnrhr^{GRIC/+}$. Control littermates from the $Bmpr1a^{+/-}$;$Gnrhr^{GRIC/+}$ × $Bmpr1a^{fl/fl}$ 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 above, $Bmpr1a^{+/-}$ 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 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 yellow fluorescent protein (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 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 LβT2 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 cKO relative to control gonadotropes (Fig. 1C).

Fertility is normal in Bmpr1a cKO mice

Eight to 10 week old control and cKO males and females were paired with wild-type C57BL/6 (Charles River) opposite sex partners for a period of six 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 (Figs. 3A and B), and pituitary *Lhb* and *Fshb* mRNA levels (Figs. 3C and D) in post-pubertal (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 LβT2 cell model (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). In 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 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, 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 TGFβ 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 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 LβT2 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 to 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 LβT2 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 (e.g., 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*$^{fl/fl}$, 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 us to be unjustified.

We pursued BMP2 and BMPR1A (by extension) because BMP2 and BMP4 were 10-fold more potent than BMP6 or BMP7 in stimulating *Fshb* transcription in LβT2 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 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 LβT2 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 TGFβ superfamily ligands should focus on activins and related proteins that signal via ACVR1B (ALK4), TGFBR1 (ALK5), and/or ACVR1C (ALK7).

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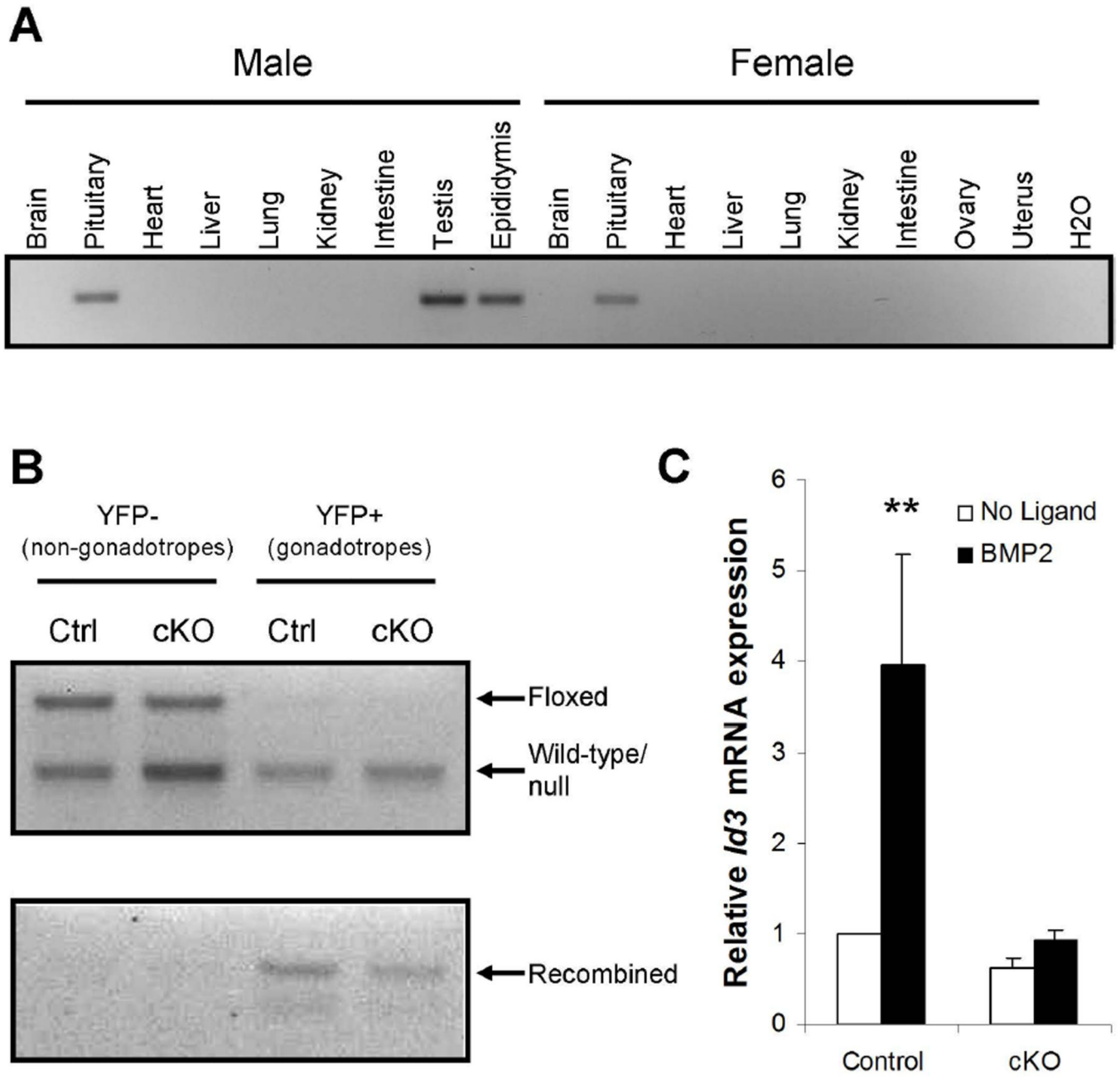

Fig. 1.

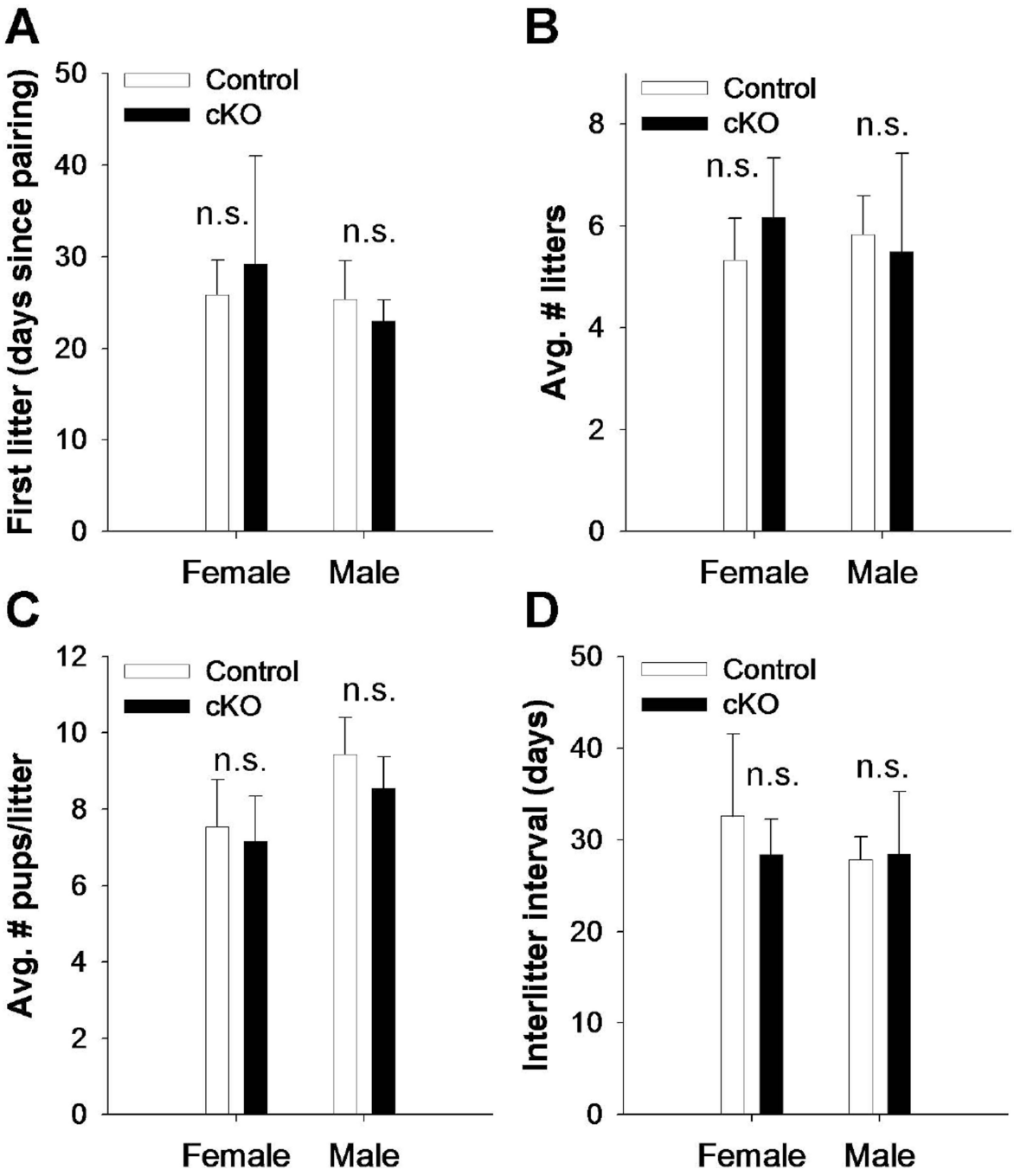

Fig. 2.

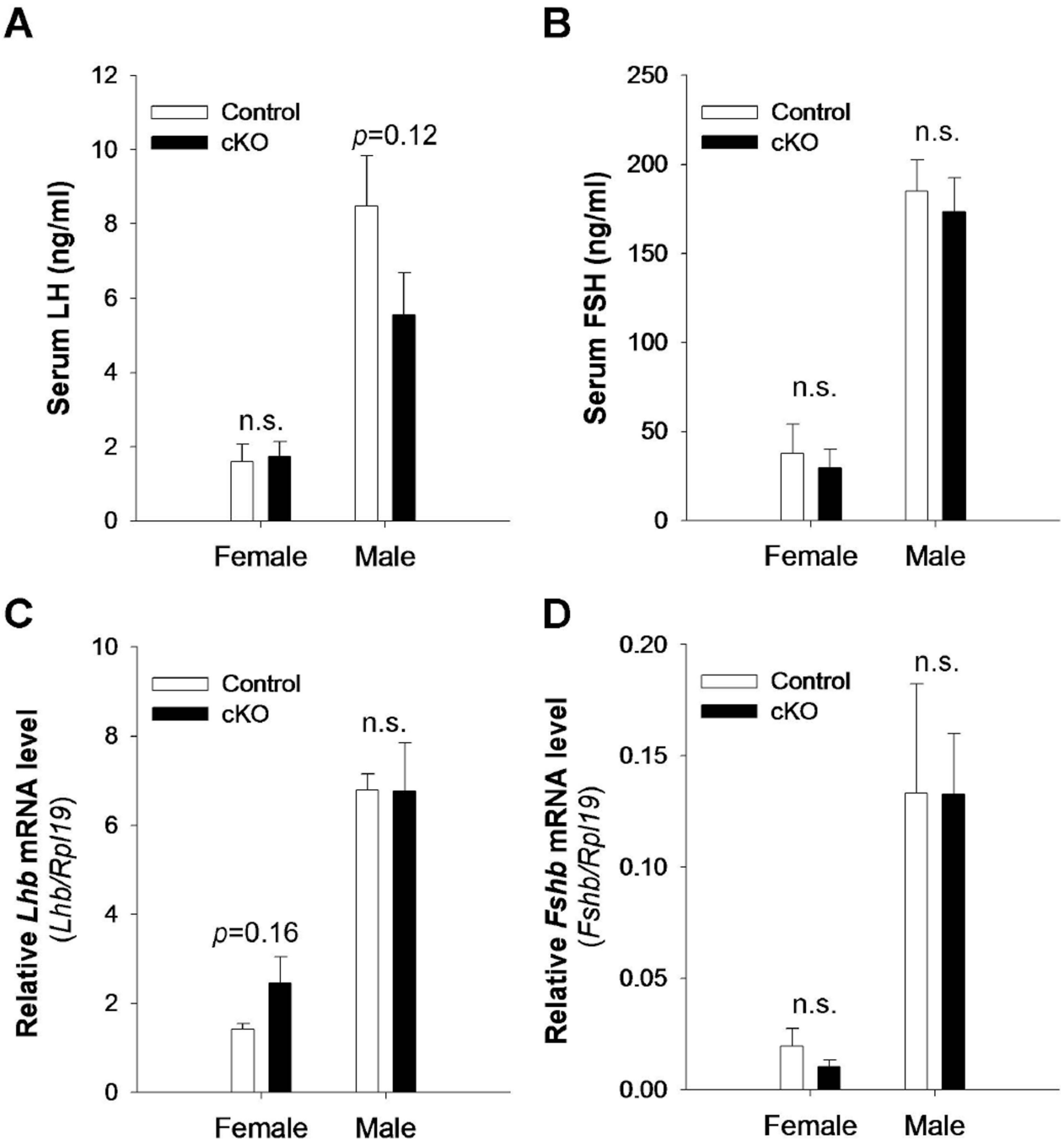

Fig. 3.

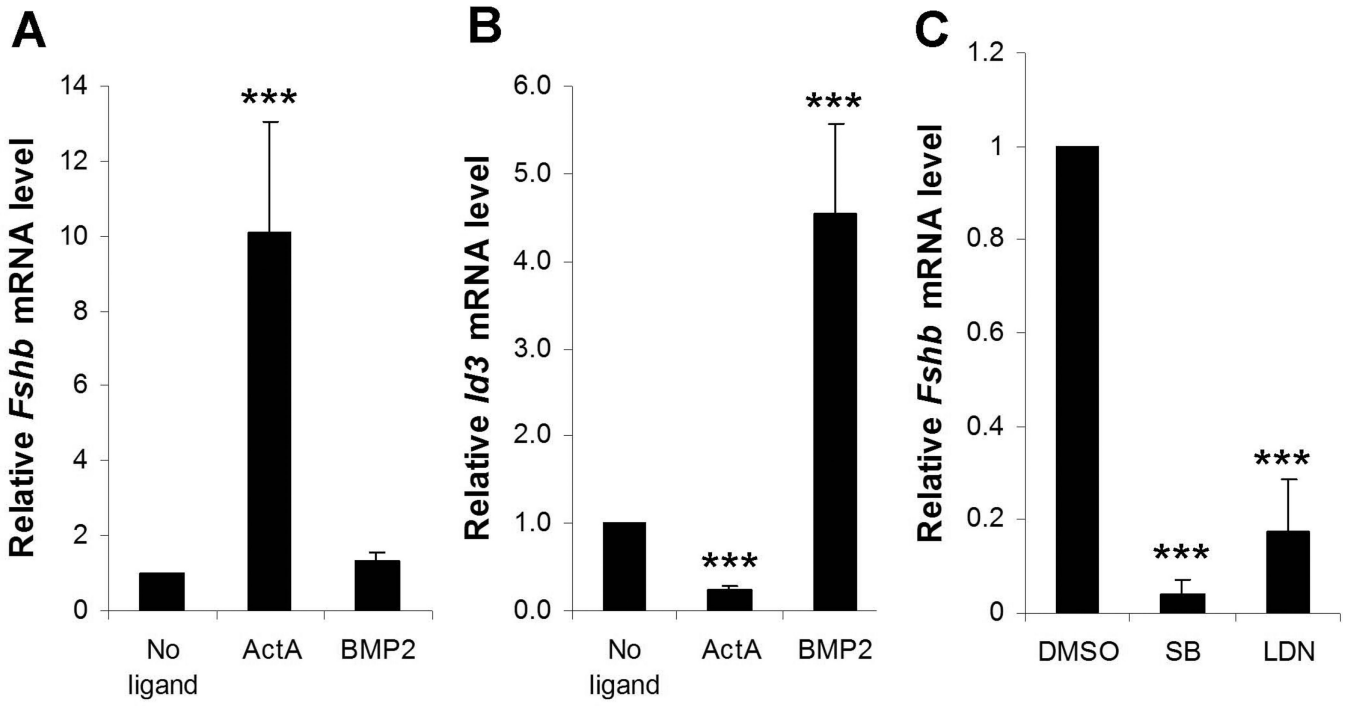

Fig. 4.

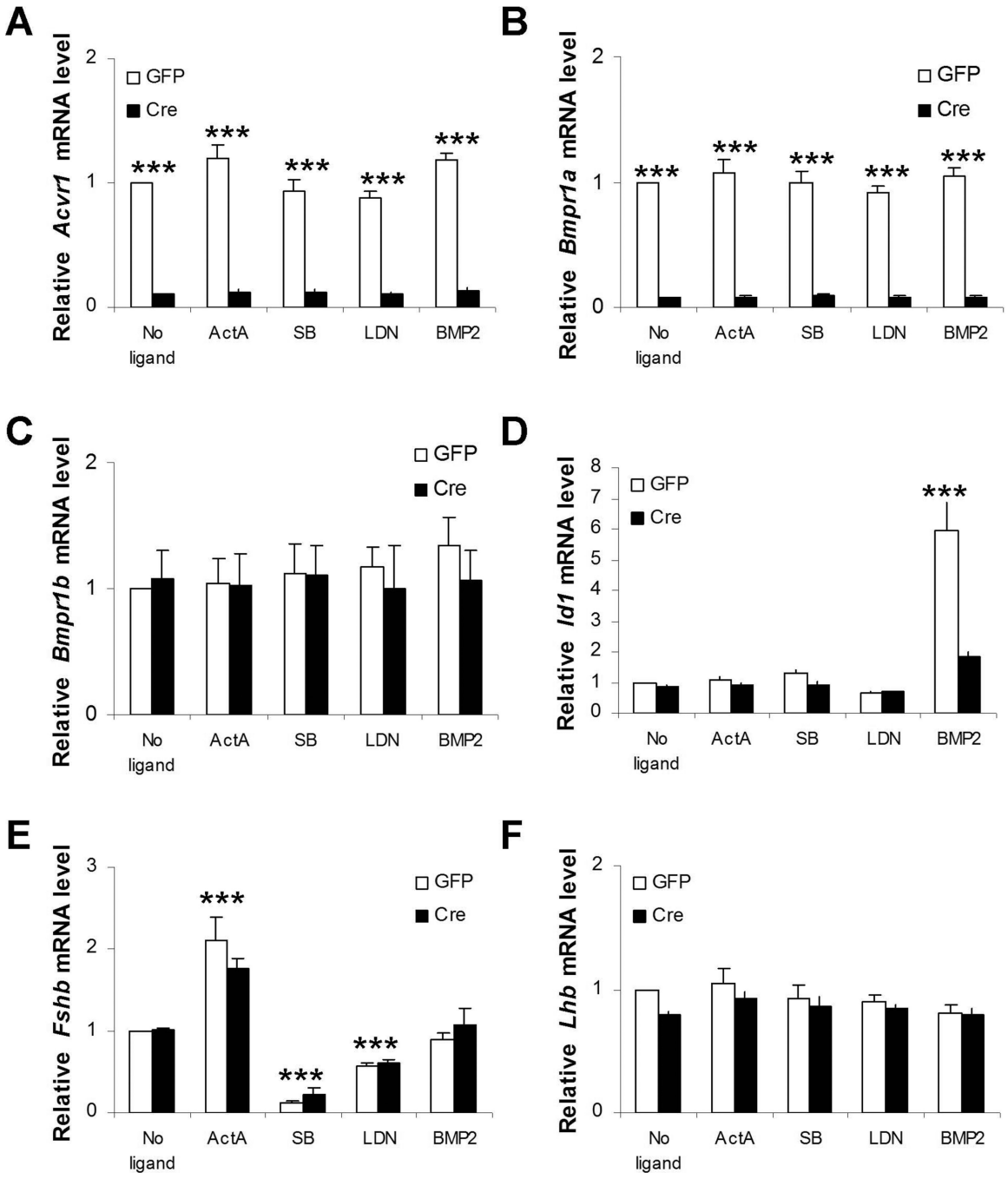

Fig. 5.

Table 1

Genotyping and RT-qPCR primers

Genotyping
Cre
Fwd: GGACATGTTCAGGGATCGCCAGGC
Rev: GCATAACCAGTGAAACAGCATTGCTG
Acvr1
Fwd: CCCCCATTGAAGGTTTAGAGAGAC
Rev: CTAAGAGCCATGACAGAGGTTG
Bmpr1a
Fwd1: CTCTGAATTTCTAGTCCACATCTGC
Fwd2: AGACTGCCTTGGGAAAAGCGC
Rev: GGACTATGGACACACAATGGC
RT-qPCR
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: TTCAGCTTGTGGATGTGCTC	

Table 2

Body and reproductive organ weights

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

Notes: Data are means ± SEM.

*, Paired organ weights. Data were analyzed with unpaired t-tests. *p* values are presented for each comparison.