Transcription factor GATA2 may potentiate follicle-stimulating hormone production in mice via induction of the BMP antagonist gremlin in gonadotrope cells

Received for publication, January 28, 2022, and in revised form, May 15, 2022. Published, Papers in Press, May 25, 2022, https://doi.org/10.1016/j.jbc.2022.102072

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Edited by Mike Shipston

Mammalian reproduction depends on the gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone, which are secreted by pituitary gonadotrope cells. The zinc-finger transcription factor GATA2 was previously implicated in FSH production in male mice; however, its mechanisms of action and role in females were not determined. To directly address GATA2 function in gonadotropes, we generated and analyzed gonadotrope-specific Gata2 KO mice using the Cre-lox system. We found that while conditional KO (cKO) males exhibited ~50% reductions in serum FSH levels and pituitary FSHβ subunit (Fshb) expression relative to controls, FSH production was apparently normal in cKO females. In addition, RNA-seq analysis of purified gonadotropes from control and cKO males revealed a profound decrease in expression of gremlin (Grem1), a bone morphogenetic protein (BMP) antagonist. We show Grem1 was expressed in gonadotropes, but not other cell lineages, in the adult male mouse pituitary. Furthermore, Gata2, Grem1, and Fshb mRNA levels were significantly higher in the pituitaries of WT males relative to females but decreased in males treated with estradiol and increased following ovariectomy in control but not cKO females. Finally, we found that recombinant gremlin stimulated Fshb expression in pituitary cultures from WT mice. Collectively, the data suggest that GATA2 promotes Grem1 expression in gonadotropes and that the gremlin protein potentiates FSH production. The mechanisms of gremlin action have not yet been established but may involve attenuation of BMP binding to activin type II receptors in gonadotropes, facilitating induction of Fshb transcription by activins or related ligands.

Gonadal function is regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH), dimeric glycoproteins produced and secreted by gonadotrope cells of the anterior pituitary gland (1, 2). LH and FSH share a common α subunit (chorionic gonadotropin alpha subunit [CGA], encoded by Cga) that is noncovalently linked to hormone-specific β subunits (LHβ and FSHβ, encoded by Lhb and Fshb, respectively). Production of the β subunits is rate-limiting in the synthesis of each hormone, making it critical to understand the transcriptional regulation of Lhb and Fshb. Expression of both β subunits is stimulated by the hypothalamic decapetide gonadotropin-releasing hormone (GnRH) (3), while Fshb is also regulated by transforming growth factor β (TGFβ) ligands (4–6). GnRH stimulates Lhb expression via the coordinated activity of the transcription factors EGR1, PITX1, and NRS5A1 (SF1) (7–13). The mechanisms through which GnRH regulates Fshb are unresolved. In contrast, TGFβ family ligands, such as the activins, stimulate Fshb expression via SMAD3, SMAD4, and FOXL2 (14–21). The zinc-finger transcription factor, GATA2, has also been implicated in the regulation of both LH and FSH production (22–25).

GATA2 was originally described as a transcription factor necessary for gonadotrope lineage specification during embryonic development (22). Gata2 mRNA is enriched in murine gonadotropes relative to other GATA factors (26) and relative to other pituitary cell lineages (27–29). Transgenic mice expressing GATA2 in pituitary cell types that normally do not express the protein show an expansion of the gonadotrope population (22). Conversely, transgenic mice expressing a dominant-negative form of GATA2 exhibit reduced expression of gonadotrope marker genes (22).

Apart from its role in pituitary development, GATA2 may also regulate the gonadotropin subunits. Conditional deletion of GATA2 in gonadotropes and thyrotropes (another pituitary cell lineage) leads to decreased FSH production in male mice (hereafter called “Gata2 pitKO” males) (24). Though serum LH levels were not measured in these animals, pituitary LHβ protein levels and seminal vesicle weights (an indirect marker of LH and testosterone production) were decreased. GATA2 can also stimulate human CGA promoter activity (23), as well as rat Lhb promoter activity and murine Lhb expression in a murine gonadotrope-like cell line, LBT2 (25). It is presently

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unclear how GATA2 regulates gonadotropin subunit expression in vivo.

In the Gata2 pitKO model, Gata2 expression was reduced in at least two pituitary cell lineages, precluding an assessment of GATA2's cell autonomous role(s) in gonadotropes. Also, GATA2 function in females was not reported (24). Here, we generated and characterized mice in which Gata2 was ablated specifically in gonadotropes. FSH production was impaired in male but not female mice. The data suggest that the bone morphogenetic protein (BMP) antagonist, gremlin, is a GATA2 target that contributes to the sex-specific regulation of FSH in mice.

Results

Generation of Gata2 conditional KO mice

To investigate the role of GATA2 in gonadotropes, we generated Gata2 conditional KOs (cKOs) by crossing floxed Gata2 mice (Gata2<sup>fl/fl</sup>) with the Cre-driver line, GRIC (Gnrh<sup>GRIC/GRIC</sup>). First, we confirmed the specificity of Gata2 deletion in the cKO animals. Gene recombination was evident in the pituitary glands in both sexes and in testes and epididymides of males (Fig. S1, A and B). This is consistent with previous studies showing exclusive Cre activity from the GRIC allele in gonadotropes and in the male germline (15, 30–34). Next, we used reverse transcription quantitative PCR (RT-qPCR), with one primer directed against the floxed exon (exon 5), to quantify the Gata2 deletion in purified gonadotropes. In cKOs of both sexes, Gata2 mRNA levels were markedly reduced in the gonadotrope-enriched population (GFP+ cells in Fig. S1, C and D) relative to the other pituitary cell lineages (e.g., thyrotropes (22, 35); Tomato+ cells in Fig. S1, C and D), confirming the deletion of exon 5 in gonadotropes. Single nuclei RNA-Seq (sn RNA-Seq: Fig. S2) further demonstrated the lack of reads over the targeted exon in Gata2 in gonadotrope cells of cKO but not control mice (Fig. S1E). Henceforth, measurements of Gata2 mRNA levels using RT-qPCR should be implicitly read as levels of intact Gata2 mRNA. Gata2 transcripts lacking exon 5 do not encode a functional GATA2 protein (24).

FSH levels are reduced in Gata2 cKO males but not females

Adult male cKO animals (Gata2<sup>fl/fl</sup>;Gnrh<sup>GRIC/GRIC</sup>) had reduced serum FSH levels relative to controls (Gata2<sup>fl/fl</sup>;Gnrh<sup>+/+</sup>) (Fig. 1A), while their serum LH was unaltered (Fig. 1B). In contrast, on the morning of estrus, female cKO animals had equivalent FSH (Fig. 1D) and LH (Fig. 1E) levels to those of controls. Consistent with the serum hormone concentrations, pituitary Fshb mRNA levels were significantly reduced in cKO males (Fig. 1C) but not females (Fig. 1F) relative to controls. Lhb mRNA levels were not significantly affected in either sex, while Cga expression showed a male-specific reduction in cKOs (Fig. 1C, C and F). According to sn RNA-Seq analyses, reductions in Fshb and Cga levels reflected a diminution in transcripts per cell rather than a decrease in the total number of gonadotropes expressing these genes (Fig. S3A). Gnrh mRNA levels were significantly elevated in cKO females but not males (p = 0.07). Full-length Gata2 mRNA levels were significantly reduced in both sexes (Fig. 1, C and F). Other pituitary lineages (mainly thyrotropes and a small subset of somatotropes) contributed to the residual Gata2 expression in cKO pituitaries (Figs. 1, C and F, S1, C, D and S3, B, C).

Reproductive organs are normal in both male and female Gata2 cKO animals

Male cKO animals showed no changes in testicular (Fig. S4A) or seminal vesicle (Fig. S4B) weights compared to littermate controls. Sperm concentrations (Fig. S4C) and testicular architecture (Fig. S4D) were also comparable between genotypes. cKO females showed normal: (1) puberty onset, as assessed by vaginal opening (Fig. S4E); (2) estrous cyclicity (Fig. S4F); (3) fertility (Fig. S4G); and (4) ovarian weights (Fig. S4H). Ovaries from both control and cKO females contained follicles at all stages (Fig. S4F).

Given normal testicular weights in the face of reduced FSH production in adult cKO males, we next assessed serum FSH levels during postnatal development. FSH was comparable between the two genotypes at 3 weeks of age. Whereas, control males showed increases in serum FSH levels from 4 to 7 weeks of age, cKO males did not (Fig. S5). Thus, FSH deficiency first occurred around the time of weaning, after testicular Sertoli cell numbers (and spermatogenic potential) are established (36).

Gata2 deletion does not increase inhibin sensitivity or follistatin expression in male pituitaries

The male-specific FSH phenotype was reminiscent of another model in which a gain-of-function (stabilizing) mutation was introduced into β-catenin (Gtmnb1) in murine gonadotropes (37). There, the male-specific FSH deficiency derived from increased pituitary sensitivity to inhibins and increased pituitary expression of follistatin (Fst) (37). Both inhibins and follistatins antagonize the actions of activins (and related TGFβ ligands), which stimulate Fshb expression.

To assess whether Gata2 cKO males had increased sensitivity to inhibins, we injected mice with anti-inhibin serum (AIS) (38–40). In our experience, control adult male mice have limited inhibin tone (40). As a result, AIS did not affect their FSH levels. In contrast, FSH levels increased in response to AIS in WT females (Fig. S6A). Neither control nor cKO males showed significantly elevated serum FSH levels 6 or 11 h after AIS injection (Fig. S6B), suggesting that inhibin sensitivity remains low in both genotypes.

With respect to potential changes in follistatin expression, we did not detect the Fst mRNA in pituitaries from either genotype by RT-qPCR or in bulk or single-nuclei datasets (data not shown). These results are consistent with other single-cell RNA-Seq data (27–29, 41). Follistatin-like 3 (encoded by Fstl3) has some overlapping functions with follistatin (42, 43). However, we did not observe a difference in Fstl3 expression between control and cKO males when assessed by RT-qPCR or sn RNA-Seq (Fig. S7, A and B).
GATA2 deletion blunts increases in gonadotropin subunit expression following gonadectomy

To begin to understand how male but not female cKOs exhibited FSH deficiency, we gonadectomized control and cKO males to investigate the potential influence of testicular factors. Following castration, both control and cKO mice showed increased serum FSH levels; however, cKO animals continued to produce less FSH than controls (Fig. 2A). Serum LH levels also increased post-castration in both genotypes, but the response was blunted in cKOs relative to controls (Fig. 2B). Pituitary Fshb (Fig. 2C), Lhb (Fig. 2D), and Cga (Fig. 2E) mRNA levels increased following gonadectomy in both genotypes but to a lesser extent in cKOs. There were nonsignificant trends for Gnrhr mRNA levels to be higher in cKOs relative to controls and to be lower in both genotypes following castration (Fig. 2F).

We conducted a similar experiment in females to assess whether ovarian factors may mask the effects of GATA2 loss in gonadotropes. Females of both genotypes showed increases in serum FSH (Fig. 3A) and LH (Fig. 3B) levels following ovariectomy. However, there was a nonsignificant trend (p = 0.08) for the FSH response to be blunted in cKO females relative to controls (Fig. 3A). LH levels were higher in cKOs compared to controls postovariectomy (Fig. 3B). Pituitary Fshb (Fig. 3C), Lhb (Fig. 3D), and Cga (Fig. 3E) mRNA levels were lower in ovariectomized cKO females relative to controls. In contrast, Gnrhr expression was increased in both sham-operated and ovariectomized cKO females relative to controls (Fig. 3F).

Estradiol administration masks some effects of GATA2 loss in males

Given that ovariectomy unmasked phenotypes in pituitaries of cKO females, we hypothesized that estradiol might contribute to sex differences observed in gonad-intact cKO mice. To begin to test this idea, we implanted castrated control and cKO males with silastic implants containing oil vehicle or
17β-estradiol (E2). E2 treatment did not significantly affect FSH levels and did not reverse the inhibitory effect of Gata2 KO (Fig. S8A). In contrast, E2 suppressed LH levels (relative to oil treatment) in both control and cKO mice and eliminated the genotype difference in serum LH (Fig. S8B). In the pituitary, E2 reduced the expression of Fshb, Lhb, and Cga in both controls and cKOs and largely eliminated the genotype differences observed in oil-treated mice (Fig. S8, C–E). Similarly, the difference in Gnrhr mRNA levels between controls and cKOs was eliminated by E2 (Fig. S8F).

GATA3 does not compensate for the loss of GATA2 in gonadotropes

We next asked whether the residual FSH production in Gata2 cKO males was enabled by GATA3 activity in gonadotropes. Indeed, GATA3 compensates for the absence of GATA2 in some contexts (24, 44). However, pituitary Gata3 mRNA levels, as assessed by RT-qPCR, were low and did not differ between controls and cKOs in either sex (Fig. S9, A and B). Gata3 mRNA was undetected in gonadotropes in sn RNA-Seq data (not shown). Nevertheless, we generated Gata2/Gata3 double cKO (dcKO) mice to investigate a potential compensatory role for GATA3. Cre-mediated recombination was efficient in both sexes (Fig. S9, C–H). The phenotypes of dcKO males were akin to those of Gata2 cKO males, with normal testicular (Fig. S9I) and seminal vesicle weights (Fig. S9J), decreased serum FSH levels (Fig. S9K), and normal LH levels (Fig. S9L). Pituitary Fshb and Cga mRNA levels were reduced, as in Gata2 cKOs (Fig. S9M). The only difference we observed between the two models was a statistically significant increase in Gnrhr levels in dcKO males relative to controls.
Though we did not systematically characterize dcKO females, their fertility was unaffected (data not shown). GATA2 is required for normal Fshb expression in adult animals

Next, we assessed whether the decrease in FSH production in males was caused by embryonic defects in pituitary development. Cre activity in GRIC mice is first observed in the pituitary at embryonic day 12.75 (30). Gata2 is expressed approximately 2 days earlier at embryonic day 10.5 (22). We did not detect differences in gonadotrope cell numbers (Fig. S10A) or in pituitary expression of Nr5a1 (a gonadotrope-specific marker) between control and cKO males (Fig. S10B).

A postnatal role for GATA2 in gonadotrope function was suggested by the emergence of FSH deficiency in cKO males after 3 weeks of age (Fig. S5). However, to rule in or out developmental effects, we used a tamoxifen-inducible gonadotrope-specific Cre-driver (16) to ablate Gata2 in adult male mice. As in Gata2 cKOs, inducible KO mice exhibited reductions in pituitary Gata2, Fshb, and Cga, and increases in Gnrhr mRNA levels (Fig. S11A). Lhb expression was unaffected (Fig. S11A). Serum FSH levels were not significantly reduced in inducible KOs, though there was a trend in this direction (p = 0.20; post-TAM in Fig. S11B).

Grem1 is downregulated in gonadotropes of cKO males

After ruling out several mechanisms to explain the sex-specific FSH impairment in cKO males, we performed bulk RNA-Seq on purified gonadotropes of male control and cKO mice to search for novel regulators. We detected the
upregulation of 114 and the downregulation of 119 transcripts in male cKO gonadotropes relative to controls (Fig. 4, A and B). The most downregulated transcript in cKO gonadotropes was Grem1, which encodes the BMP antagonist gremlin. We also observed reduced Grem1 expression in gonadotropes of cKO males using sn RNA-Seq (Fig. 4C). Notably, Grem1 and Gata2 were expressed at higher levels in pituitaries of gonad-intact (SHAM) WT males than females (Fig. 4, D and E and (26)), but the abundance of both transcripts increased following ovariectomy in females (Fig. 4, D and E). Thus, Gata2 and Grem1 expression appear to be positively correlated.

Figure 4. Grem1 is repressed in gonadotropes of Gata2 cKO males. A, volcano plot with the log2 fold changes in gene expression in gonadotropes isolated from control and Gata2 cKO males on the x-axis and the statistical significance (−log10 p-value) on the y-axis. Differentially expressed genes were selected based on fold change ≥ 0.5 (i.e., log2 fold change ≥ 0.5 in either direction; the mean gene expression [normalized log2 counts per million] of one of the two groups ≥ 4.0; and FDR ≤ 0.05). The gene symbols of a subset of differentially expressed genes are displayed. B, heatmap of bulk RNA-Seq expression for the 233 genes selected from panel (A) that were differentially regulated between gonadotropes isolated from control (two columns on the left) and Gata2 cKO (two columns on the right) mice. Gene expression is shown in normalized log2 counts per million. The scale is shown on the right of the heatmap, with warm colors showing upregulation and cold colors downregulation. C, violin plot showing Grem1 expression (from sn RNA-Seq) in control and cKO male gonadotropes (N = 3 per genotype). Each dot represents a single cell. D and E, pituitary gene expression profiles in sham-operated (SHAM) and gonadectomized (GDX) control males and females (D) Grem1 and (E) Gata2. In (D and E), expression levels were normalized to those in SHAM females (collected on random estrous cycle stages). mRNA levels were measured by RT-qPCRs and normalized to the housekeeping gene Rpl19. Results were analyzed by two-way ANOVA, followed by post hoc Holm–Sidak multiple comparison, ***p < 0.001 (comparison between sexes); #p < 0.05 (comparison between surgeries within sex). cKO, conditional KO; FDR, false discovery rate; FSH, follicle-stimulating hormone; RT-qPCR, reverse transcription quantitative PCR; sn RNA-Seq, single nuclei RNA-Seq.
To gain greater insight into the mechanisms underlying Grem1 expression, we assessed chromatin accessibility using an assay for transposase-accessible chromatin using sequencing (ATAC-seq). The Grem1 promoter was accessible (open) in gonadotropes and thyrotropes (but not other pituitary cell lineages; Fig. 5A) of control males. There were also two coaccessible regions upstream (5') of the gene uniquely in gonadotropes (green and red boxed peaks in Fig. 5A). All these regions were predominantly closed in gonadotropes of cKO males (Fig. 5A, bottom track, and Fig. 5B, top three tracks) and WT females (Fig. 5B, lowest three tracks). Thus, accessibility of the Grem1 locus is restricted when GATA2 is lost or low in gonadotropes.

**Pituitary Grem1 expression is regulated by GATA2 and estradiol**

As BMPs were previously implicated in FSH regulation (45–49) and considering the aforementioned results, we further investigated Grem1. First, we confirmed the RNA-Seq results by RT-qPCR; pituitary Grem1 expression was decreased by 95% in gonad-intact cKO males (Fig. 6A). Pituitary Grem1 mRNA levels were also reduced when Gata2 was ablated in gonadotropes of adult males using the tamoxifen-inducible Cre-driver (Fig. S1A). Grem1 expression was not affected by castration in control or cKO males (Fig. 6A). The pattern of pituitary Gata2 expression paralleled that of Grem1 (Fig. 6B). As noted previously, the residual Gata2 mRNA in cKO mice reflects expression in other pituitary lineages (Figs. 6B, S1C and S3).

In females, ovariectomy increased pituitary Grem1 (Fig. 6C) and Gata2 (Fig. 6D) expression in controls (flxed without Cre), replicating what we observed in WT animals (Fig. 4, D and E). In cKO females, however, the postovariectomy increase in Grem1 was blocked (Fig. 6C). There was a small increase in Gata2 levels in ovariectomized cKO females, perhaps reflecting gene expression changes in other pituitary cell types (see Fig. S1D).

In light of the data in ovariectomized females, we analyzed pituitary Grem1 and Gata2 mRNA levels in castrated control and cKO males treated with E2 (or vehicle). E2 significantly decreased pituitary Grem1 and Gata2 expression in control males (relative to oil-treated males; Fig. 6E, F and F). However, they were not decreased fully to the levels seen in cKO mice (Fig. 6, E and F).

**Gremlin increases Fshb mRNA levels in primary pituitary cells**

Finally, we treated WT male pituitary cultures with recombinant gremlin (or vehicle) to assess effects, if any, on Fshb mRNA expression. Recombinant gremlin concentration-dependently increased Fshb, but not Lhb, mRNA levels (Fig. 7A). Id1, Id2, and Id3 expression was decreased following gremlin treatment (Fig. 7B), consistent with the expected antagonism of BMP signaling.

**Discussion**

We generated Gata2 cKO mice to investigate GATA2 function in gonadotrope cells. Our results both confirm and significantly extend earlier observations. Akin to what was previously reported in Gata2 pitKO mice (24), FSH levels are significantly reduced in cKO relative to control males. In contrast, FSH is normal in gonad-intact cKO females. We were able to rule out changes in inhibin sensitivity, increases in pituitary Fst or Fstl3 expression, and alterations in pituitary development as the causes of impaired FSH production in males. Instead, sex differences in GATA2-dependent gremlin expression and action in gonadotropes may explain the selective FSH deficiency in cKO males.

**GATA2 regulates Grem1 expression in a sex-specific manner**

Our results indicate that GATA2 is expressed at higher levels in male relative to female pituitaries, at least in gonad-intact mice. Ovariectomy increases pituitary Gata2 mRNA levels in females, whereas 17β-estradiol decreases pituitary Gata2 expression in males. Thus, sex differences in estrogen levels may explain, at least in part, both higher Gata2 levels in males and the sex-specific effects of Gata2 ablation. Indeed, it is only following ovariectomy that phenotypes are observed in cKO females. How estrogens regulate Gata2 in gonadotropes is not yet clear as we do not observe any estrogen response elements in the Gata2 promoter, nor did we identify putative enhancers for Gata2 in our sn ATAC-seq datasets.

The data also converge to indicate that GATA2 regulates Grem1 expression in gonadotropes. Not only are Grem1 mRNA levels dramatically downregulated in gonadotropes of cKO males but both Gata2 and Grem1 levels are also higher in males than females and are coordinateively increased post-ovariectomy in the latter. It is notable, however, that ovariectomy does not elevate Gata2 or Grem1 expression to levels seen in males, suggesting that ovarian factors alone may not account for all the observed sex differences. We also have not determined how GATA2 regulates Grem1 expression. Chromatin accessibility of the Grem1 gene is reduced when GATA2 levels are low (as in gonad-intact females or in cKO males), but we did not find canonical GATA-binding sites (WGATAR) in the presumptive promoter region (though several sites are observed within intron 1 in Grem1, Fig. 5A). Interestingly, we observed two coaccessible regions upstream of the Grem1 gene in gonadotropes of control males (Fig. 5A). These potential enhancer regions contain several canonical GATA-binding sites and are compacted in gonadotropes of gonad-intact females and cKO males. Future studies should determine whether GATA2 acts via these cis-elements to regulate Grem1 transcription.

**Is gremlin a novel regulator of FSH production?**

The data suggest, but do not definitively demonstrate, that gremlin loss explains FSH deficiency in Gata2 cKO mice. In vitro, recombinant gremlin dose-dependently stimulates Fshb expression, indicating that the protein can positively regulate FSH production. Nevertheless, the in vivo evidence we present is currently correlative. When pituitary Gata2 and Grem1 mRNA levels are high (as in control males), so too is Fshb expression. Conversely, pituitary Grem1 expression is low.
Figure 5. The Grem1 gene is accessible in control male gonadotropes. A, chromatin accessibility in and around the Grem1 gene in a control male mouse pituitary was assessed by sn ATAC-seq. Different pituitary cell types are labeled at the right. Regions of open chromatin, as reflected by peaks of sequence reads, in gonadotrope cells are boxed. The black box corresponds to exon 1 and proximal promoter. Two 5′ regions with coaccessibility in gonadotropes (potential enhancers) are boxed in green and red. Consensus GATA-binding sequences (WGATAR) are highlighted in gray within blowups of the boxed regions. Note the track at the bottom showing sn ATAC-seq data for gonadotropes from a cKO male.

B, chromatin accessibility around the first exon/promoter of Grem1 in gonadotropes in three individual cKO males (top three tracks), three individual control males (middle three tracks), and three individual WT females (bottom three tracks). cKO, conditional KO; sn ATAC-seq, single nuclei assay for transposase-accessible chromatin using sequencing.

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J. Biol. Chem. (2022) 298(7) 102072
or absent in females and their FSH levels are significantly lower than in males. Postovariectomy, both Grem1 and Fshb expression increase in control (but not cKO) female pituitaries. Therefore, gremlin might play previously unappreciated roles in regulating both sex differences and ovariectomy-dependent increases in FSH production in mice. To conclusively demonstrate that gremlin positively regulates FSH in vivo, Grem1 could be ablated in gonadotropes (e.g., using Grem1fx/fx (50) crossed to GRIC mice). Here, we predict that FSH levels would be reduced in males and in ovariectomized females.

How might gremlin regulate FSH production?

Even if gremlin stimulates FSH synthesis, our data do not currently explain its mechanism of action. Gremlin is a BMP antagonist, and BMPs were previously reported to regulate Fshb expression in different contexts (45, 48, 51). Nevertheless, mice lacking canonical BMP type II (BMPR2) (34) or type I receptors (ACVR1 and BMPR1A) (52) in gonadotropes produce FSH normally. Therefore, BMPs do not appear to signal directly in gonadotropes to regulate FSH production in mice. It is possible, however, that BMPs and, by extension, gremlin regulate FSH indirectly; perhaps by modulating activin-like signaling.

Activins are generally considered the canonical TGFβ ligands that stimulate Fshb expression (53–55). Mice lacking the activin type II receptors, ACVR2A and ACVR2B, in gonadotropes are FSH deficient (32, 56). Some BMPs can also bind these receptors (57–59). It is therefore possible that BMPs compete with activins (or related ligands) for binding to ACVR2A/B on the surface of gonadotropes. Though BMP binding to these receptors might not provoke signaling that
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Figure 7. Gremlin increases Fshb expression in murine primary pituitary cultures. Pituitary cells were isolated from WT adult males (8–10 weeks of age) on day 1. On day 2, cells were treated with vehicle or increasing concentrations of gremlin-1 (0.02, 0.05, 0.1, 0.2, and 0.5 μg/ml) for 6 h. A, Fshb, Lhb, and β2) id, id2, and id3 mRNA levels were determined by RT-qPCR. Rp119 was used for normalization. Data represent the mean ± SEM of three independent experiments. Results were analyzed by one-way ANOVA, followed by post hoc Holm–Sidak multiple comparison. *p < 0.05, **p < 0.01, ***p < 0.001. RT-qPCR, reverse transcription quantitative PCR.

directly affects FSH production, it could nevertheless antagonize signaling by activins. If this model is accurate, then gremlin may play a role in FSH synthesis by bone-mimicking BMPs in the pituitary, providing a permissive environment for BMPs in the pituitary, providing a permissive environment for gremlin may play a role in FSH synthesis by bioneutralizing activin-like ligands to bind ACVR2A/B to induce FSH production. However, the latter effect is modest and may explain why the Lhb impairment in our study was only observed following gonadectomy, when Lhb expression is at its highest levels.

GATA2 regulates gonadotropin subunit expression

Although FSH deficiency was the clearest phenotype in cKO males, GATA2 appears to have pleiotropic effects on gonadotropin subunit gene expression. Pituitary Lhb expression was reduced in cKO males and in cKO females postgonadectomy. GATA2 can induce the rat Lhb promoter in vitro (25) and Gata2 knockout in LβT2 cells decreases endogenous Lhb expression (25), suggesting a role for GATA2 in Lhb transcription. However, in contrast, the increase in Cga mRNA levels in gonad-intact cKO males, but not females, might reflect a difference in sensitivity to the loss of GATA2, as GATA2 is more highly expressed in gonadotropes of males than females. Consistent with this idea, Gata2 and Cga expression increase in ovariectomized control females. In contrast, the increase in Cga is significantly blunted in ovariectomized cKO females. Chromatin immunoprecipitation studies would be valuable in determining whether and how Lhb and Cga are targeted by GATA2.

Conclusions

We propose that GATA2 promotes FSH synthesis in mice by regulating the expression of gremlin, which attenuates BMP antagonism of activin-like signaling in gonadotropes. Future studies are needed to determine: (1) how GATA2 regulates Grem1 expression, (2) whether the effects of Gata2 ablation on FSH are caused, in whole or in part, by gremlin loss in vivo, and (3) how estradiol regulates Gata2 expression in gonadotropes. In addition, it will be important to establish whether gremlin regulation of FSH is mouse-specific or conserved across species. According to recent RNA-Seq analyses, Grem1 appears to be expressed in thyrrotropes rather than gonadotropes in rats (29). Nevertheless, rat thyrotropes express Gata2 (29), which could drive Grem1 expression in that lineage, and gremlin (or another BMP antagonist) could act on gonadotropes in a paracrine manner in this species. GATA2 function in human pituitary is unclear; however, gonadotrope-derived pituitary adenomas express the protein (62). RNA-Seq analyses indicate that pediatric (but not adult or geriatric) human pituitaries express GATA2 but not GREM1 (63). GREM1 is not detected in any pituitary cell lineage across sexes and ages, although the related GREM2 is expressed by somatotropes.
(63). As in rats, this may imply paracrine, rather than autocrine regulation of gonadotrope function by gremlin proteins or perhaps other soluble BMP antagonists in humans.

Experimental procedures

Animals

Gata2<sup>fx/fx</sup> and Gnrhr<sup>IRES-Cre/IRES-Cre</sup> (GRIC) mice were described previously (24, 31). Gata2<sup>fx/fx</sup> males (MMRC, stock 030290-MU, RRID: MMRRC_030290-MU) were crossed with GRIC females to generate Gata2<sup>fx/+;</sup>Gnrhr<sup>GRIC/+</sup> progeny. Gata2<sup>fx/+;</sup>Gnrhr<sup>GRIC/+</sup> females were then crossed to Gata2<sup>fx/fx</sup> males to generate Gata2<sup>fx/fx;</sup>Gnrhr<sup>+</sup>/+ (control) and Gata2<sup>fx/fx;</sup>Gnrhr<sup>GRIC/+</sup> (cKO) animals. Eventually, given that cKO females had normal fertility, Gata2<sup>fx/fx;</sup>Gnrhr<sup>GRIC/+</sup> females were mated to Gata2<sup>fx/fx</sup> males so that all progeny could be used for experiments (50% control, 50% cKO). Genotyping and assessment of genomic recombination were conducted as previously described (52) (primers listed in Table 1). Animals were housed ad libitum on a 12L:12D cycle (lights on at 7:00, lights off at 19:00). All animal experiments were performed in accordance with institutional and federal guidelines and were approved by the Downtown-A Facility Animal Care Committee of McGill University (Protocol 5204).

Fluorescence-activated cell sorting

To purify gonadotropes by fluorescence-activated cell sorting (FACS), we crossed Gata2<sup>fx/</sup> animals with Gt(ROSA26)<sup>ACTB-tdTomato-EGFP</sup> mice (mTmG/mTmG, stock 007676 from Jackson Laboratories) to generate Gata2<sup>fx/</sup>Rosa26<sup>mTmG/mTmG</sup> males, which were then crossed to Gata2<sup>fx/</sup>Gnrhr<sup>GRIC/+</sup> females to generate Gata2<sup>fx/</sup>Gnrhr<sup>GRIC/+;Rosa26<sup>mTmG</sup></sup> and Gata2<sup>fx/</sup>Gnrhr<sup>GRIC/+;Rosa26<sup>mTmG</sup></sup> females. Controls for FACS were generated by crossing Rosa26<sup>mTmG</sup> and Gnrhr<sup>GRIC</sup> mice to produce Gata2<sup>+</sup>Gnrhr<sup>GRIC/+;Rosa26<sup>mTmG</sup></sup> progeny. FACS was performed at the Flow Cytometry Core at the Montreal Clinical Research Institute (IRCM).

Protocols for pituitary cell dispersion and cell sorting were adapted from previous publications (15, 64). Briefly, we dispersed two to three pituitary glands per digestion tube per genotype and per sex, each in technical duplicates. Each sample was then sorted individually, after which the purified...
cells for a given genotype and sex were pooled. On average, 3.6 × 10^6 enhanced GFP–positive (gonadotropes) and 1.1 × 10^6 tdTomato-positive cells (nongonadotropes) were obtained from each pool (4–6 mice per pool).

**Blood collection**

Blood was collected from 8- to 10-week-old males or 9- to 10-week-old females (at 7:00 on estrus morning) by cardiac puncture. Blood was allowed to clot for 30 to 60 min at room temperature (RT) and was then spun down at 3000 rpm for 10 min to collect serum. Sera were stored at −80 °C until assayed for LH and FSH.

**Hormone analyses**

Unless otherwise specified, serum FSH levels were assessed using Milliplex kits (Millipore; MPTMAG-49K, custom-made for FSH only) following the manufacturer’s instructions (limit of detection (LOD): 23.7 pg/ml; dynamic range: 61.0 pg/ml to 250,000 pg/ml; limit of quantification (LOQ): 61.0 pg/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%). Some FSH measurements (Fig. S8A) were done using an in-house ELISA described in (65) (LOD: 0.046 ng/ml; dynamic range: 0.046 ng/ml to 2 ng/ml; LOQ: 0.48 ng/ml; intra-assay CV < 15%). Serum LH levels were measured using an in-house sandwich ELISA as previously described (15, 66) (LOD: 0.117 ng/ml; dynamic range: 0.017 ng/ml to 30 ng/ml; LOQ: 0.516 ng/ml; intra-assay CV < 15%).

Reverse transcription and quantitative PCR

Pituitary glands were dissected from control and cKO animals (same age as described previously, unless otherwise specified), snap-frozen in liquid nitrogen, and stored at −80 °C. Pituitaries were homogenized in TRIzol reagent (15596018; ThermoFisher Scientific), and total RNA was extracted following the manufacturer’s guidelines. For FACS samples, total RNA was extracted using Total RNA Mini Kits (Geneaid; RB300).

Reverse transcription was performed as previously described (66) using Moloney murine leukemia virus reverse transcriptase (0000172807; Promega) and random hexamers (0000184865; Promega). Quantitative PCR runs were conducted on a Corbett Rotor-Gene 600 instrument (Corbett Life Science) using EvaGreen quantitative PCR Mastermix (ABMMmix-S-XL; Diamed) and the primers listed in **Table 2**. Expression levels of genes of interest were determined using the 2^−ΔΔCt method (67) and ribosomal protein L19 (Rpl19) for normalization. All primers were validated for efficiency and specificity.

**Table 2**

| Gene | Primer sequence |
|------|-----------------|
| Gga | TCCCTCAAAAAGTCCAOAGGC |
| Forward | GAAGAGAAATGAGAATAGCAG |
| Reverse | GTCGGGCTACTGCTACTACT |
| Cgh | CGAGCAATCTTGCTGTCG |
| Forward | CCTGTGCTGCGACGAGTGA |
| Reverse | ACTACCTGTAATGCTTGT |
| Gata3 | GCCATACGTGGAAGGTT |
| Forward | AGATTCCCCACTGCGGTTTC |
| Reverse | TTCGCCTACCTCGTTCCT |
| Gnrhr | CACGGGTATAGAAGAAGAAG |
| Forward | AAGAGCGGACACTCATGCTACA |
| Reverse | GGAAACTCTTGCGGCTGGCAAG |
| Estradiol implants | TTTGATGCGGTGTCCTG |
| Forward | TTTTGTGAGGGGGGCGG |
| Reverse | CAGGCTACGTGAGGTCG |
| Id2 | GTGCTACTTGATCACG |
| Forward | GCCATACGTGGAAGGTT |
| Reverse | ATTCAGATGCCTGCAAGGAC |
| Id3 | TTACAGATGCCTGCAAGGAC |
| Forward | TCGCCCGTGTCCTGACG |
| Reverse | TATCGGCAAGGATTCT |
| Lhb | CGAGGGGTGGTGTCG |
| Forward | ACCAAGGTGTTAGAAGAAG |
| Reverse | ACTGGGTCGCTCCCT |
| Nr5a1 | TTACAGATGCCTGCAAGGAC |
| Forward | TCGCCCGTGTCCTGACG |
| Reverse | TATCGGCAAGGATTCT |
| Rpl19 | CGGAAATCCAAGAAGATTGA |
| Forward | TCGGCTTGGATGTTG |
| Reverse | CTACAGATGCCTGCAAGGAC |

**Gonadectomy**

Males and females were gonadectomized at between 7 and 8 weeks of age in compliance with the standard operating procedures at McGill University (standard operating procedures 206 and 207). Briefly, for males, an incision was made at the midline of the scrotum at the level of the skin and then the tunica. Each testis was pushed out and cauterized. The wound was then closed with sutures and veterinary glue. For females, an incision was made at the midline of the mid-dorsum of the animal. A small incision was made in the muscle above each ovary; the ovary was then pulled out and cauterized. All incisions were closed by sutures. In the case of sham-operated animals, all the procedures were the same, except that the gonads were not cauterized. Unless specified otherwise, gonadectomized animals were left to recover for 2 weeks, at which point they were euthanized to collect the pituitary gland and serum (from cardiac puncture blood). For these experiments, sham-operated females were euthanized on metestrus/diestrus.
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Analysis of bulk RNA-Seq data

Raw data fastq files were aligned to the GENCODE mm10 genome using STAR (69). Gene expression was quantified with featureCounts (70). The raw data fastq files and the final gene expression matrix were deposited to GEO (see details later). Differential expression analysis was performed using Bioconductor (71) package limma (72) under R version 3.6.1 (RCoreTeam, http://www.R-project.org/). After filtering for low expression, gene expression was analyzed using a voom method (73) and compared between control and cKO mice. Benjamin–Hochberg correction (74) for multiple comparisons testing was applied. The differentially expressed genes were determined with (i) the false discovery rate of ≤ 0.05; (ii) the log2 fold change ≥ 0.5 in either direction; and (iii) the mean gene expression in log2 of counts per million ≥ 4.0 in at least one of the two groups.

Validation of RNA-Seq results was conducted by RT-qPCR on new FACS samples. FACS was performed as described previously, and ~70,000 gonadotropes were obtained from 12 to 16 control and cKO animals. Such a high yield was necessary to detect some rare transcripts by RT-qPCR.

Nuclei isolation from pituitaries

Individual pituitaries were processed as described in (41). Briefly, on ice, snap-frozen control and Gata2 cKO pituitaries were thawed and prepared based on a modified protocol from (75). Each pituitary was homogenized individually in a Dounce glass homogenizer (1 ml, VWR; catalog no.: # 71000-514), and the homogenate was filtered through a 40 μm cell strainer before proceeding for gradient centrifugation (SW41 rotor at 14,400g; 4 °C; 25 min). Nuclei were collected from the interphase, washed, resuspended either in 1X nuclei dilution buffer for sn ATAC-seq (10X Genomics) or in 1X PBS/0.04% bovine serum albumin for sn RNA-Seq, and counted (Invitrogen Countess II) before proceeding for sn ATAC-seq assay.

sn ATAC-seq assay

After nuclei were counted, sn ATAC-seq was performed following the Chromium Single Cell ATAC Reagent Kits V1 User Guide (10X Genomics). Transposition was performed in 10 μl at 37 °C for 60 min on at least 1000 nuclei, before loading of the Chromium Chip E (PN-2000121). Barcoding was performed in the emulsion (12 cycles) following the manufacturer’s recommendations. Libraries were indexed for multiplexing (Chromium i7 Sample Index N, Set A kit PN-3000262) and pooled for sequencing. Sequencing was performed at the New York Genome Center Novaseq 6000 (Illumina).

sn ATAC-seq analysis of individual samples

sn ATAC-seq data were processed using Cell Ranger-ATAC pipeline version 1.2.0. Sequencing results from samples processed in multiple wells were combined using the Cell Ranger-ATAC aggr function. After an initial quality control, we used Signac version 1.2.0 to perform clustering analysis on the aggregated samples. As part of our quality control, we removed cell barcodes that were outliers in their percentage of reads in peaks, their number of peak region fragments, their transcriptional start site enrichment score, their ratio of reads aligned to blacklist regions of the genome, or their nucleosome signal. The exact cutoffs varied from sample to sample, as both sample quality and sequencing depth can affect the aforementioned metrics, but typical acceptable values are the following: percentage read in peaks > 25%, number of peak region fragments > 500, transcriptional start site enrichment score > 2, blacklist ratio < 0.05, nucleosome signal < 4. After clustering, we used the 10X Genomics’ Loupe Browser to visualize and explore chromatin accessibility and annotated cell types (clusters) based on cut-site pileup sums at promoter regions of known cell type markers (41).
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Gonadotrope accessibility tracks were created with igvtools 2.3.32 from the list of fragment cut-sites (fragments file) and cell barcodes belonging to the gonadotrope cluster, using a window size of 200 base pairs per cut-site. Tracks were normalized to both the number of gonadotropes and to the median number of fragments per gonadotrope in each sample. The KO-1 mouse was arbitrarily chosen as the reference sample for the median number of fragments, to which all other samples were scaled, as it nicely features a peak accessibility of 1.0 over the Actb gene. Tracks were visualized in IGV 2.7.2 (76).

Primary pituitary cultures

Pituitaries from 8- to 10-week-old WT male mice (C57BL/6) were extracted and dispersed as previously described (32, 34, 66). On day 1, cells were seeded in 48-well plates (250,000–400,000 cells/well) and cultured in M199 medium (31100-035; Invitrogen) supplemented with 10% fetal bovine serum (10438026; ThermoFisher Scientific). The next day, cells were treated (in serum-free medium) for 6 h with increasing concentrations of gremlin (120-42; PeproTech): 0, 0.02, 0.05, 0.1, 0.2, and 0.5 μg/ml. After the treatment, RNA was extracted using a Total RNA Mini kit (FA32808-PS; Geneaid), and RNA was processed for RT-qPCR as described previously.

Statistical analysis

Data were analyzed on GraphPad Prism 8 (Graphpad Software Inc) using Student t-tests or two-way ANOVA followed by post hoc Holm–Sidak multiple comparison, as specified in figure legends. Results were considered statistically significant when p < 0.05.

Data availability

Most data are available in the article. RNA-seq and ATAC-seq data were deposited in GEO; accession numbers GSE189915 (bulk RNA-seq), GSE190060 (sn RNA-seq), GSE190064 (sn ATAC-seq), GSE190066 (compiled). The sn ATAC-seq for the WT females were previously deposited in GEO as GSE151960.

Supporting information—This article contains supporting information. References used (16, 27, 38–41, 65, 77–81).

Acknowledgments—We would like to thank Dr Maxime Bouchard (McGill University) for generously providing the floxed Gata3 animals. We acknowledge the New York Genome Center for sequencing. This work was supported in part through the computational and data resources and staff expertise provided by Scientific Computing at the Icahn School of Medicine at Mount Sinai.

Author contributions—G. S. and D. J. B. conceptualization; G. S., L. O., F. R. Z., and M. Z. methodology; M. Z. software; G. S., L. O., F. R. Z., M. Z., and Y. G. formal analysis; G. S., L. O., E. B., X. Z., Y. W., F. R. Z., N. M., N. S., M. A. A., and V. N. investigation; U. B. resources; M. Z. data curation; G. S. and D. J. B. writing—original draft; G. S., L. O., E. B., X. Z., Y. W., U. B., F. R. Z., M. Z., N. M., N. S., M. A. A., V. N., Y. G., S. C. S., and D. J. B. writing—review & editing; G. S., L. O., and M. Z. visualization; S. C. S. and D. J. B. supervision; G. S., S. C. S., and D. J. B. project administration; J. B., S. C. S. and D. J. B. funding acquisition.

Funding and additional information—This work was supported by the Canadian Institutes of Health Research, Canada (operating grants PJT-162343 and -169184 to D. J. B., and Doctoral Research Award 152308 to G. S.), the Natural Sciences and Engineering Research Council of Canada, Canada (2015-05178 to D. J. B. and Doctoral Scholarship to E. B.), Fonds de Recherche du Québec – Santé, Canada (fellowship number 31338 to G. S.), and a National Institute of Health (NIH), United States Grant DK46943 (S. C. S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AIS, anti-inhibin serum; ATAC-seq, assay for transposase-accessible chromatin using sequencing; CGA, chorionic gonadotropin alpha subunit; cKO, conditional KO; FACS, fluorescence-activated cell sorting; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; LOD, limit of detection; LOQ, limit of quantification; RT-qPCR, reverse transcription quantitative PCR; sn RNA-Seq, single nucleic RNA-Seq.

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