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The Forkhead Transcription Factor, FOXP3, Is Required for Normal Pituitary Gonadotropin Expression in Mice[1]

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ABSTRACT

The hypothalamic-pituitary-gonadal axis is central to normal reproductive function. This pathway begins with the release of gonadotropin-releasing hormone in systematic pulses by the hypothalamus. Gonadotropin-releasing hormone is bound by receptors on gonadotroph cells in the anterior pituitary gland and stimulates the synthesis and secretion of luteinizing hormone and, to some extent, follicle-stimulating hormone. Once stimulated by these glycoprotein hormones, the gonads begin gametogenesis and the synthesis of sex hormones. In humans, mutations of the forkhead transcription factor, *FOXP3*, lead to an autoimmune disorder known as immunodysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome. Mice with a mutation in the *Foxp3* gene have a similar autoimmune syndrome and are infertile. To understand why FOXP3 is required for reproductive function, we are investigating the reproductive phenotype of *Foxp3* mutant mice (*Foxp3*$^{sf/Y}$). Although the gonadotroph cells appear to be intact in *Foxp3*$^{sf/Y}$ mice, luteinizing hormone beta (*Lhb*) and follicle-stimulating hormone beta (*Fshb*) expression are significantly decreased, demonstrating that these mice exhibit a hypogonadotropic hypogonadism. Hypothalamic expression of gonadotropin-releasing hormone is not significantly decreased in *Foxp3*$^{sf/Y}$ males. Treatment of *Foxp3*$^{sf/Y}$ males with a gonadotropin-releasing hormone receptor agonist does not rescue expression of *Lhb* or *Fshb*. Interestingly, we do not detect *Foxp3* expression in the pituitary or hypothalamus, suggesting that the infertility seen in *Foxp3*$^{sf/Y}$ males is a secondary effect, possibly due to loss of FOXP3 in immune cells. Pituitary expression of glycoprotein hormone alpha (*Cga*) and prolactin (*Prl*) are significantly reduced in *Foxp3*$^{sf/Y}$ males, whereas the precursor for adrenocorticotropic hormone, pro-opiomelanocortin (*Pomc*), is increased. Human patients diagnosed with IPEX often exhibit thyroiditis due to destruction of the thyroid gland by autoimmune cells. We find that *Foxp3*$^{sf/Y}$ mice have elevated expression of thyroid-stimulating hormone beta (*Tshb*), suggesting that they may suffer from thyroiditis as well. Expression of the pituitary transcription factors, *Pitx1, Pitx2, Lhx3,* and *Egr1,* is normal; however, expression of *Foxl2* and *Gata2* is elevated. These data are the first to demonstrate a defect at the pituitary level in the absence of FOXP3, which contributes to the infertility observed in mice with *Foxp3* loss of function mutations.

forkhead, FOXP3, gonadotropins, pituitary

INTRODUCTION

The Pituitary Gland

Normal reproductive function depends on the hypothalamic-pituitary-gonadal axis. Pulsatile secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates GnRH receptors (GnRHRs) on the surface of pituitary gonadotroph cells, increasing the number of GnRHRs and stimulating synthesis and secretion of luteinizing hormone (LH) and, to some extent, follicle-stimulating hormone (FSH). FSH secretion is dependent on the presence of activin. The degree of GnRH stimulation depends on both the quantity of GnRH released by the hypothalamus and the concentration of GnRHRs on the surface of gonadotroph cells. LH and FSH are dimeric hormones comprised of a common glycoprotein hormone α subunit (αGSU), but it is the unique β subunits that render their unique functions. FSH acts on Sertoli cells, structures that form a blood-testis barrier functioning as a filter that permits only certain substances to reach spermatocytes [1]. In males, LH stimulation of Leydig cells results in secretion of testosterone that is essential for spermatogenesis. In females, a surge of LH initiates ovarian follicle luteinization and meiotic maturation of the oocyte [2, 3]. Transcriptional regulation of the LH β (*Lhb*) gene involves steroidogenic factor 1 (SF1, *Nr5a1*), early growth response factor 1 (EGR1), and pituitary transcription factor 1 (PITX1) [4]. Gonadal steroids can then negatively feed back at the levels of the pituitary and the hypothalamus.

Infertility in the United States

Male infertility is a component in half of infertile couples, and in approximately one third of cases male infertility is the sole cause [5]. Epidemiological studies suggest that approximately 80 million people worldwide are infertile [6]. Although the etiology is largely unknown, some necessary genes have been identified. LH and its receptor are among these. LH acts on the gonads to facilitate production of testosterone, estrogen, and progesterone [4, 7] and is essential for spermatogenesis and ovulation. Low LH levels can cause reduced libido, bone density, and muscle mass, whereas overexpression of LH increases the risk of ovarian and testicular tumors [7]. Thus, understanding how LH production is regulated is key for treating a number of human pathologies.

The Forkhead Transcription Factor, FOXP3

The ''forkhead'' family is classified based on a conserved DNA-binding domain or forkhead domain. This family has

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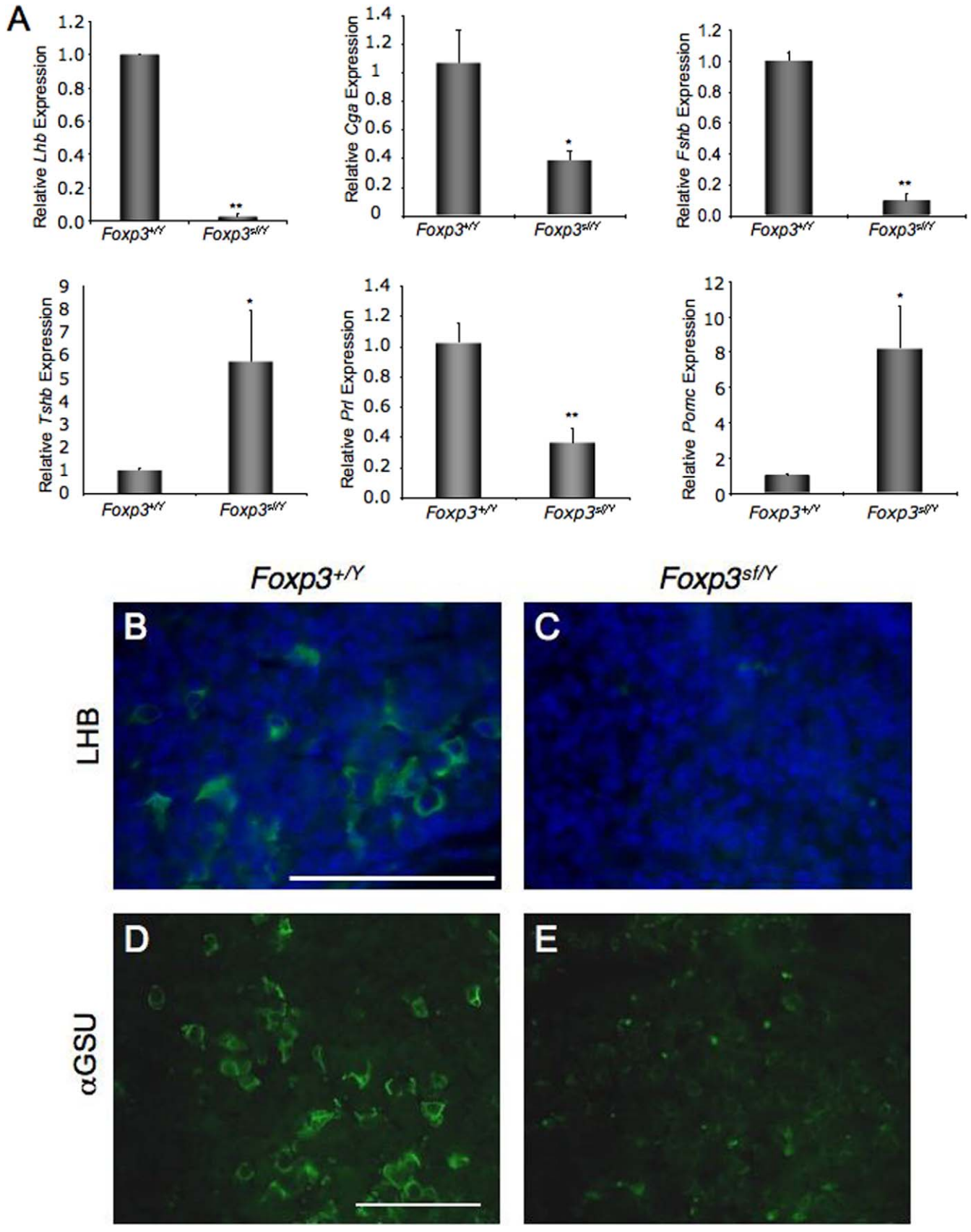

FIG. 1. Several pituitary hormones are misexpressed in *Foxp3*$^{sf/Y}$ mice. **A**) Expression levels of *Lhb*, *Cga*, *Fshb*, and *Prl* were significantly reduced in *Foxp3*$^{sf/Y}$ mice as compared to wild-type male littermate controls. *Tshb* and *Pomc* expression were significantly increased. Real-time RT-PCR experiments were performed using pituitary from 6- to 9- (*Lhb*) or 6- (*Cga, Fshb, Prl, Tshb, Pomc*) wk-old male mice. Expression level was calculated by the $\Delta\Delta C_T$ method and represents expression relative to the average ΔC_T of wild-type samples. Data are expressed as mean ± SEM of four animals per group. The data were analyzed by Student *t*-test to determine significant difference between wild-type and *Foxp3*$^{sf/Y}$ mice (*$P < 0.05$; **$P < 0.01$). **B**–**E**) Pituitary from 6-wk-old *Foxp3*$^{sf/Y}$ mice and wild-type littermates was sectioned coronally and analyzed by immunohistochemistry. **B**, **C**) Cytoplasmic LHB

over 100 members and is present in organisms as simple as yeast and as complex as humans [8]. The forkhead factor, FOXP3, is constitutively expressed in $CD4^+CD25^+$ regulatory T (Treg) cells. FOXP3 plays important roles in the differentiation and function of Treg cells [9]. The gene encoding FOXP3 is located on the X chromosome in humans and mice. Mutations in the human *FOXP3* gene result in an autoimmune syndrome referred to as immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX). Symptoms include diarrhea, eczema, hemolytic anemia, diabetes mellitus, and thyroid autoimmunity leading to hypothyroidism [10]. Death often occurs during the first years of life [10].

Mutations in the Murine Foxp3 *Gene*

Foxp3 is expressed in Treg cells as well as in thymic, breast, and prostate epithelial cells [11, 12]. A spontaneously occurring mutation, referred to as scurfy (*sf*), results in an IPEX-like syndrome in mice. This mutation has been mapped to the *Foxp3* gene and consists of a 2-bp insertion causing a frame shift. This codes for a premature stop codon, producing a truncated, nonfunctional protein [13]. Interestingly, affected males ($Foxp3^{sf/Y}$) are infertile [14, 15].

MATERIALS AND METHODS

Mice

Foxp3 mutant mice were purchased from the Jackson Laboratory (www.jax.org) and maintained on a C57BL/6J background. $Foxp3^{sf/Y}$ male mice were left with dams to increase survival time. Mice were maintained in a 12L:12D cycle. To genotype mice, we used a Custom Taqman SNP Genotyping Assay (Applied Biosystems, www.appliedbiosystems.com) according to manufacturer's instructions. Male mice were used for all studies.

All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals or the Southern Illinois University Animal Care and Use Committee. All experiments were conducted in accord with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

Histology and Immunohistochemistry

Pituitaries were dissected and fixed for 20 min in 4% paraformaldehyde in PBS (pH 7.2). All samples were washed in PBS, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections (5 μm) were deparaffinized in xylene, rehydrated through a series of graded ethanol washes, and stained in hematoxylin (Fisher Scientific, www.fishersci.com) and eosin (Sigma, www.sigmaaldrich.com) or used for immunohistochemistry. To visualize LHB, and αGSU in the pituitary, slides were deparaffinized in xylene and incubated for 1 h at room temperature with an antibody directed against LHB (1:500; NHPP, www.humc.edu/hormones) or αGSU (1:150; NHPP). Slides were then incubated with an anti-guinea pig or anti-rabbit secondary antibody, respectively, and conjugated to fluorescein (FITC; 1:100 dilution; Jackson ImmunoResearch Laboratories, Inc., www.jacksonimmuno.com) for 30 min at room temperature. Following a 5-min incubation with water, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (167 nM; Molecular Probes, www.invitrogen.com). Digital images of pituitary sections were captured with a Leica DM 5000B fluorescent microscope and Retiga 2000R digital camera. FITC and DAPI section pictures were merged using Adobe Photoshop CS3.

RT-PCR

Pituitaries were dissected from mice at and stored in RNAlater (Ambion, Inc., www.ambion.com) at −20°C. Hypothalami from C57BL/6 mice at 6 wk of age were excised from 1 mm rostral of the optic chiasm to 1 mm caudal of the optic chiasm and stored in RNAlater at −20°C. Total RNA was isolated with the RNAqueous-Micro kit (Ambion, Inc.) according to manufacturer's directions. RNA concentrations were determined by spectrophotometry. RNA was treated with DNase I and DNase inactivating reagent from the TURBO DNase-free kit (Ambion, Inc.) as per manufacturer's instructions. We synthesized cDNA using ImPromII reagents and random primers (Promega, www.promega.com).

Real-time RT-PCR was performed on a CFX96 Real Time System (BioRad, www.bio-rad.com). Amplification of *Lhb* was accomplished with 0.2 μM primers (5′-ATC ACC TTC ACC ACC AGC ATC TGT, 5′-TGA GGG CTA CAG GAA AGG AGA CTA) and SYBR green master mix (BioRad). The internal control used was β-globin (*Hbb-b1*; 5′-ACATTGGC ATGGCTTTGTTT, 5′-GTTTGCTCCAACCAACTGCT) because it is not changed significantly in wild-type as compared to scurfy animals. Ten nanograms of cDNA was used in a 15-μl reaction volume. Samples and controls were run in triplicate. No-template controls and no-reverse transcriptase controls were used to assure the absence of contamination and efficacy of the DNase treatment, respectively. Amplification was achieved by the following protocol: 95°C for 3 min, 40 cycles of 95°C for 10 sec, 57°C for 1 min, plate read. Melt curve analysis was performed to ensure that no primer-dimer amplification occurred. Amplification of all other amplicons was performed using Taqman Gene Expression Assays (Applied Biosystems) as per manufacturer's instructions. For all real-time experiments, at least three individuals were included in each group. All experiments were performed in triplicate. Data were analyzed by the $\Delta\Delta C_T$ method [16, 17]. The values for $\Delta\Delta C_T$ were calculated by subtracting the average ΔC_T of controls (either wild-type for Figs. 1, 2, 4, and 5, A and B, or NaCl-treated wild-type controls for Fig. 5, C and D) from the ΔC_T for each sample.

To visualize *Foxp3* expression in pituitary, hypothalamus, and thymus, quantitative PCR was performed using primers for *Foxp3* (5′-ATC TCC TGG ATG AGA AAG GCA AGG-3′, 5′-AGA GCT CTT GTC CAT TGA GGC CA -3′) or *Actb* (5′-ACA TTG GCA TGG CTT TGT TT-3′, 5′-GTT TGC TCC AAC CAA CTG CT-3′) at 0.2 μM (see Fig. 3A). Ten nanograms of cDNA was used in a 25 μl reaction volume with Go Taq Green Master Mix (Promega). Amplification was achieved by the following protocol: 95°C for 3 min, 40 cycles of 95°C for 10 sec, 57°C for 1 min. Products were visualized by gel electrophoresis (see Fig. 3A). Taqman probes were used in real-time RT-PCR to demonstrate the integrity of the pituitary and hypothalamic RNA. We measured *Gnrh* as a positive control for the integrity of hypothalamic RNA and *Foxl2* as a positive control for the integrity of pituitary RNA. We measured β-actin (*Actb*) as an internal control. The resulting products were visualized by gel electrophoresis (see Fig. 3, B and C).

D-ala-GnRH Treatment

Mice were injected with 1 ng of the GnRH analog (D-Ala-6-GnRH) i.p. in 100 μl 0.15 M NaCl every 2 h for 48 h [18]. Two hours after the last injection, mice were euthanized and pituitary was collected. Total RNA was isolated and real-time RT-PCR was performed to measure *Lhb* expression as described above.

Statistical Analysis

All results are expressed as mean ± SEM. Data were analyzed by Student *t*-test using Microsoft Excel. *P* values less than 0.05 were considered significant (*). *P* values less than 0.01 were considered very significant (**).

RESULTS

Pituitary Hormone Expression Levels in $Foxp3^{sf/Y}$ *Males*

$Foxp3^{sf/Y}$ mice are hypogonadal and infertile [14, 15]. To begin to understand why $Foxp3^{sf/Y}$ mice are infertile, we analyzed LH production in these mice. The *Foxp3* gene is on the X chromosome and $Foxp3^{sf/Y}$ males are infertile; thus, it is difficult to obtain $Foxp3^{sf/sf}$ females. For these reasons, the following studies used only male mice. Using real-time RT-PCR, we found that mRNA levels of *Lhb* were reduced in 6- to

immunoreactivity (green) is apparent in the wild-type pituitary (**B**), but is not detected in pituitary from $Foxp3^{sf/Y}$ mice (**C**). All cell nuclei are stained with DAPI (blue). **D**, **E**) Cytoplasmic αGSU immunoreactivity is apparent in the wild-type pituitary (**D**), but is reduced in pituitary from $Foxp3^{sf/Y}$ mice (**E**). Original magnification ×630 (**B**, **C**) and ×400 (**D**, **E**); bars = 100 μm.

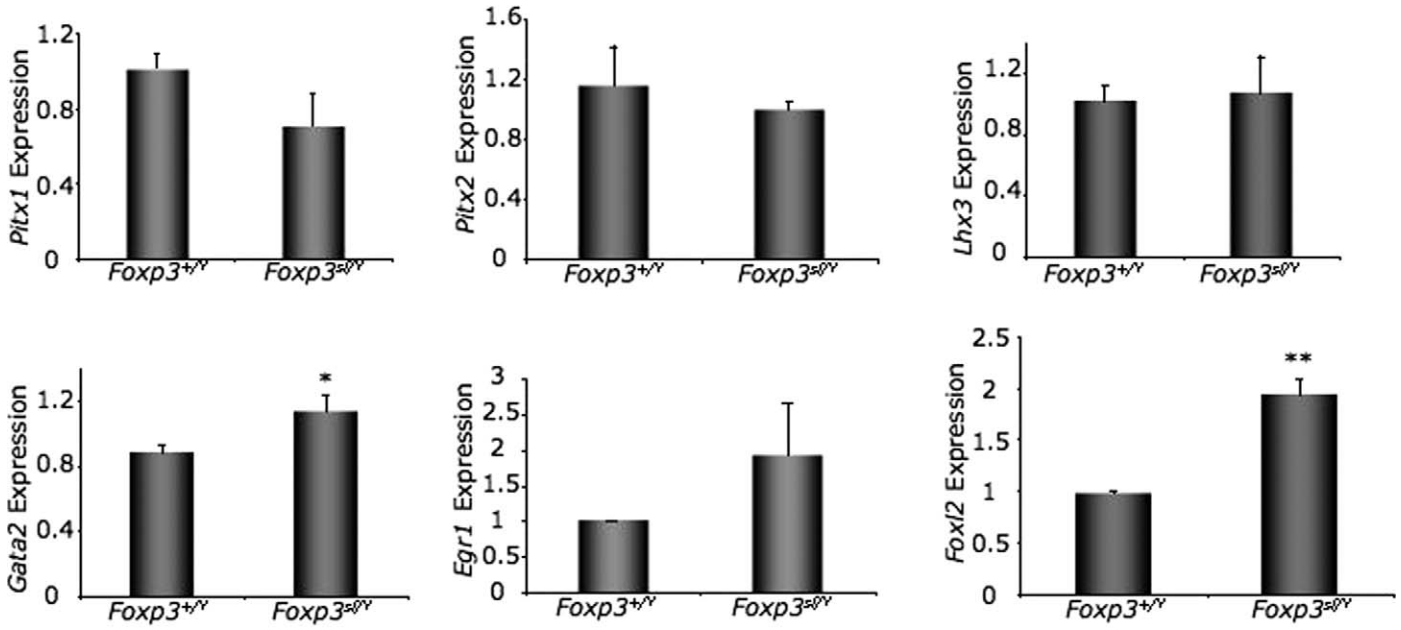

FIG. 2. Pituitary expression of some transcription factors is altered in *Foxp3*$^{sf/Y}$ mice. Pituitary expression levels of the transcription factors *Pitx1*, *Pitx2*, *Lhx3*, and *Egr1* is not significantly different in *Foxp3*$^{sf/Y}$ mice as compared to wild-type littermates. However, expression of *Gata2* and *Foxl2* is significantly increased in *Foxp3*$^{sf/Y}$ mice. Real-time RT-PCR was performed on pituitary from 6-wk-old male mice. Expression level was calculated by the $\Delta\Delta C_T$ method and represents expression relative to the average ΔC_T of samples from *Foxp3*$^{+/Y}$ mice. Data are expressed as mean ± SEM of four animals per group. The data were analyzed by Student *t*-test to determine significant difference between wild-type and *Foxp3*$^{sf/Y}$ mice (*$P < 0.05$; **$P < 0.01$).

9-wk-old *Foxp3*$^{sf/Y}$ mice (Fig. 1A). *Lhb* expression was also reduced at 3 wk of age, but not at the day before birth (data not shown). We performed immunohistochemistry on pituitary from 6-wk-old *Foxp3*$^{sf/Y}$ and *Foxp3*$^{+/Y}$ mice. We found that LHB protein was not detected in the pituitaries of *Foxp3*$^{sf/Y}$ mice (Fig. 1, B and C). These data suggested that the hypogonadism these mice exhibited was hypogonadotropic in nature.

We next investigated whether hormones other than *Lhb* were misexpressed in *Foxp3*$^{sf/Y}$ male mice. Using real-time RT-PCR to measure expression levels in 6-wk-old male mice, we found that the gonadotropin subunits, *Fshb*, and the common α subunit (*Cga*) were significantly decreased in *Foxp3*$^{sf/Y}$ male mice as compared to normal male littermates (Fig. 1A). This likely contributes to the hypogonadotropic hypogonadism exhibited by these mice. Prolactin (*Prl*) expression was also significantly reduced (Fig. 1A), whereas growth hormone (*Gh*) expression was unchanged (data not shown) in *Foxp3*$^{sf/Y}$ male mice as compared to normal male littermates. *Pomc* expression was significantly increased, possibly because of the stress these animals experienced as a result of their illness. Finally, *Tshb* expression was significantly increased (Fig. 1A). Human patients with IPEX often exhibit thyroiditis due to destruction of the thyroid gland by autoimmune cells [19]. Thus, the elevated *Tshb* expression in *Foxp3*$^{sf/Y}$ male mice is consistent with a lack of negative feedback from thyroid hormone due to possible thyroiditis. Immunoreactivity for αGSU was reduced in *Foxp3*$^{sf/Y}$ as compared to wild-type male littermates (Fig. 1, D and E). We did not detect any cells in the pituitary that exhibit normal immunoreactivity for αGSU. However, immu-

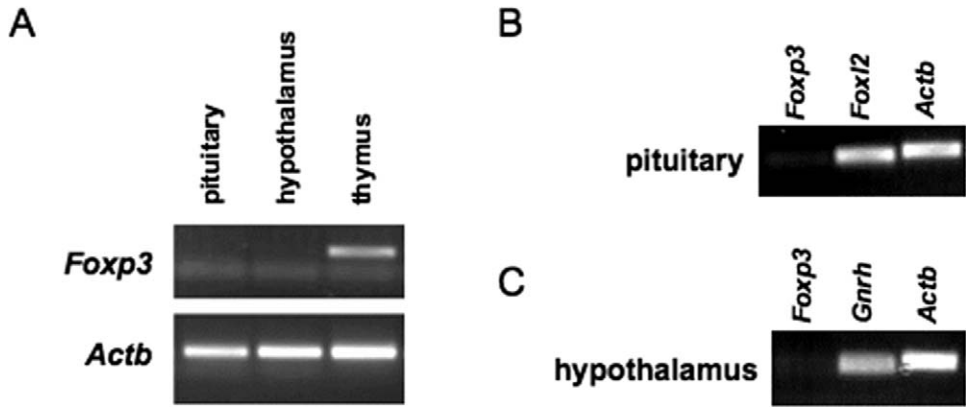

FIG. 3. *Foxp3* mRNA is not detected in adult mouse pituitary or hypothalamus. *Foxp3* expression levels were measured in pituitary (**A**, **B**) and hypothalamus (**A**, **C**) from adult C57BL/6J male mice. *Foxp3* expression in thymus was used as a positive control for our *Foxp3* assay (**A**). *Foxl2* and *Gnrh* expression was used as a positive control for the integrity of the RNA from pituitary (**B**) and hypothalamus (**C**), respectively. Products from PCR (**A**) and real-time PCR (**B**, **C**) were visualized by agarose gel electrophoresis.

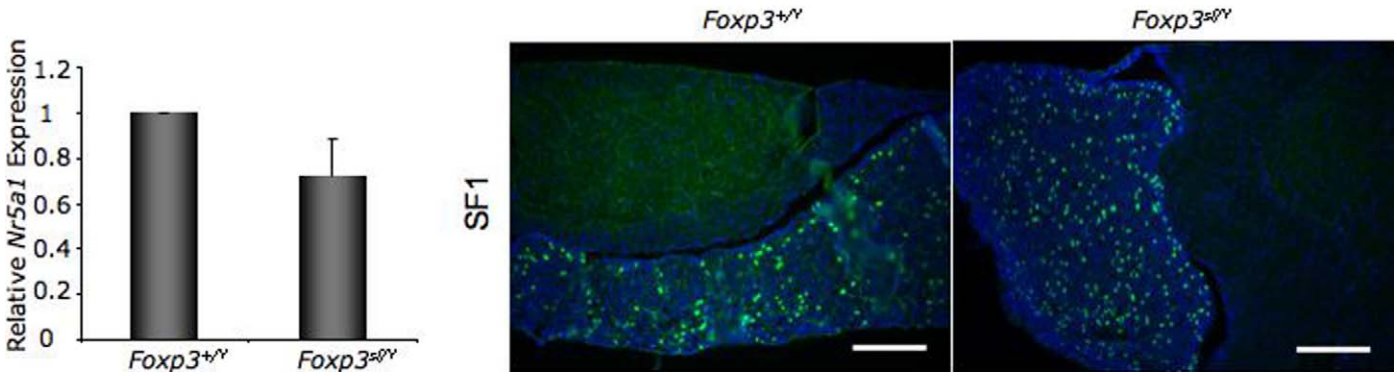

FIG. 4. Gonadotroph cells are present in pituitary from *Foxp3*$^{sf/Y}$ mice. Expression of the gene encoding SF1, *Nr5a1*, is not significantly reduced in *Foxp3*$^{sf/Y}$ mice at 6 wk of age. Expression level was calculated by the $\Delta\Delta C_T$ method and represents expression relative to the average ΔC_T of *Foxp3*$^{+/Y}$ mouse pituitary. Data are expressed as mean ± SEM of at least four animals per group and were analyzed by Student *t*-test. Pituitary sections were analyzed by immunohistochemistry for SF1 (green), which will mark gonadotroph cells, but not other pituitary cell types. All cell nuclei are stained with DAPI (blue). Pituitary from *Foxp3*$^{sf/Y}$ mice contains many SF1-positive cells, suggesting that gonadotroph cells in *Foxp3*$^{sf/Y}$ mice are intact. Original magnification ×200; bars = 100 μm.

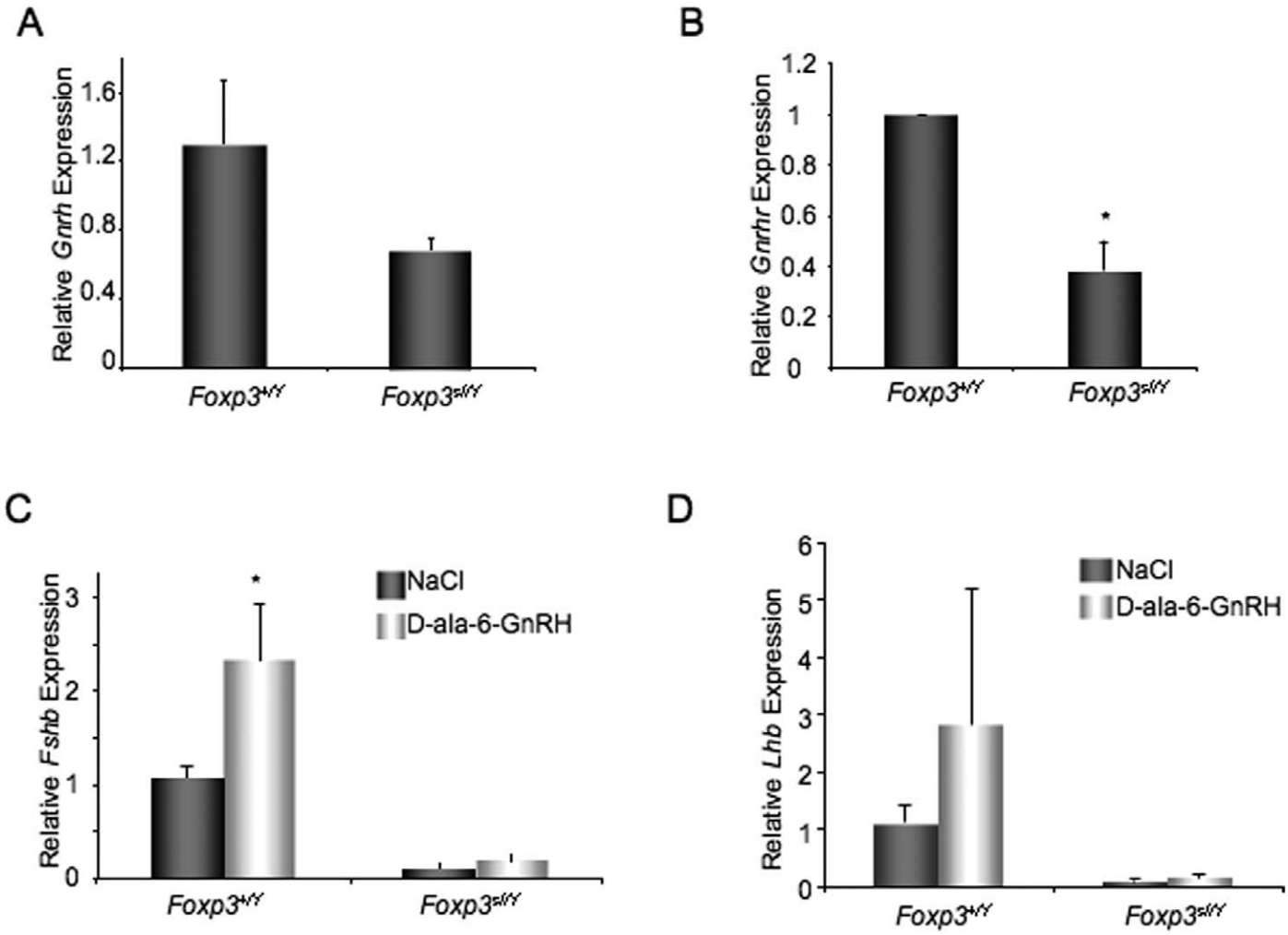

FIG. 5. GnRH treatment does not rescue *Lhb* expression in *Foxp3*$^{sf/Y}$ mice. **A**) Hypothalamic *Gnrh* expression is not significantly reduced in 6- to 9-wk-old *Foxp3*$^{sf/Y}$ mice as compared to wild-type male littermates. **B**) Expression of pituitary *Gnrhr* is significantly reduced (*$P < 0.05$) in 6- to 9-wk-old *Foxp3*$^{sf/Y}$ mice. **C**) Treatment of 6- to 9-wk-old male mice with the GnRH receptor agonist D-ala-6-GnRH significantly increases *Fshb* expression in wild-type male controls (*$P < 0.05$), but does not significantly increase *Fshb* expression in *Foxp3*$^{sf/Y}$ mice. **D**) Treatment of 6- to 9-wk-old male mice with D-ala-6-GnRH does not significantly increase *Lhb* expression in *Foxp3*$^{sf/Y}$ mice. Expression level was calculated by the $\Delta\Delta C_T$ method. Data are expressed as mean ± SEM of at least four animals per group and were analyzed by Student *t*-test. Values represent expression relative to the average ΔC_T of samples from *Foxp3*$^{+/Y}$ pituitary (**A**, **B**), or average ΔC_T of NaCl-treated *Foxp3*$^{+/Y}$ (**C**, **D**).

nohistochemistry for TSHB showed no obvious difference in the number of thyrotrope cells in *Foxp3*$^{sf/Y}$ mice (data not shown). Together these data suggested that thyrotrope cells were not producing normal amounts of αGSU or biologically active TSH.

Pituitary Transcription Factors

Several transcription factors are important for pituitary hormone expression. For example, PITX1 and EGR1 are important for stimulating expression of *Lhb* [20–23]. Mice homozygous for a hypomorphic allele of *Pitx2* lack gonadotroph cells, have a decreased number of somatotrope and thyrotrope cells, and have little or no expression of the gonadotroph transcription factors *Gata2* and *Egr1* [24, 25]. Loss of LHX3 results in an inability to produce all anterior lobe pituitary hormones except ACTH [26, 27]. Finally, FOXL2 stimulates expression of GnRH receptor (*Gnrhr*), *Cga*, and *Fshb* [28–32]. We found that expression of *Pitx1*, *Pitx2*, *Lhx3*, and *Egr1* was not different in *Foxp3*$^{sf/Y}$ male mice at 6 wk of age compared to normal male littermate controls (Fig. 2). *Gata2* and *Foxl2* expression was significantly increased in *Foxp3*$^{sf/Y}$ male mice as compared to normal male littermates, possibly in an attempt to compensate for decreased gonadotropin production (Fig. 2).

Foxp3 *Expression in Pituitary*

We next sought to understand why *Lhb* expression was reduced in *Foxp3*$^{sf/Y}$ male mice as compared to normal mice. The simplest explanation is that FOXP3 directly stimulates *Lhb* expression, which would require FOXP3 to be present in the adult pituitary. To determine if *Foxp3* is expressed in the adult male mouse pituitary, we performed RT-PCR. We isolated pituitary from male mice at 6 wk of age. Real-time RT-PCR was performed to measure *Foxp3* expression levels, using *Foxp3* expression in thymus as a positive control for the *Foxp3* primer/probe. Ct values for *Foxp3* in pituitary were consistently at background levels ranging from 36 to 40 or were undetectable (data not shown). Products were then visualized by agarose gel electrophoresis. *Foxp3* expression was detectable in thymus, but not in pituitary (Fig. 3A). *Foxl2* expression in each pituitary sample served as a positive control for RNA/cDNA integrity. Although *Foxl2* expression was detected in pituitary, *Foxp3* was not (Fig. 3B). Based on these data, we concluded that *Foxp3* was not expressed in the pituitary and that the reduction in *Lhb* expression seen in *Foxp3*$^{sf/Y}$ mice was a secondary effect due to loss of *Foxp3* in some other organ.

Gonadotroph Cells in Foxp3$^{sf/Y}$ *Mice*

Foxp3$^{sf/Y}$ mice suffer from autoimmunity. In the pancreas this results in destruction of β cells, leading to diabetes [33]. We investigated whether gonadotroph cells are intact in *Foxp3*$^{sf/Y}$ mice to determine if the loss of *Lhb* production is due to destruction of gonadotroph cells. To determine if gonadotroph cells are intact in pituitary glands from adult *Foxp3*$^{sf/Y}$ mice, we performed RT-PCR for SF1 (*Nr5a1*), which is expressed in gonadotroph cells but not in other pituitary cell types. Expression of *Nr5a1* was not different in *Foxp3*$^{sf/Y}$ male mice as compared to normal male littermates (Fig. 4). We confirmed this result with immunohistochemistry. Many SF1-positive cells were present in pituitary from *Foxp3*$^{sf/Y}$ mice (Fig. 4). These data demonstrated that gonadotroph cells were present in pituitary glands of *Foxp3*$^{sf/Y}$ mice and had not been phagocytized by the immune system.

Foxp3 *Expression in Hypothalamus*

Pituitary production of LH is intimately tied to hypothalamic function. One possible explanation is that loss of LH production is due to a hypothalamic defect. To determine if *Foxp3* is expressed in the hypothalamus, we performed RT-PCR for *Foxp3* with hypothalamic tissue. We excised hypothalami from C57BL/6 mice at 6–9 wk of age, 1 mm rostral of the optic chiasm to 2 mm caudal of the optic chiasm, to obtain GnRH neurons. Using RT-PCR, we measured *Foxp3* mRNA levels. Thymus RNA was used as a positive control for the *Foxp3* Taqman primer/probe and *Gnrh* RNA as a positive control for the integrity of the hypothalamic RNA. Hypothalamic expression of *Foxp3* was at background levels with Ct values ranging from 35 to undetectable (data not shown). Products were visualized by agarose gel electrophoresis. We did not detect *Foxp3* expression in hypothalamic tissue, indicating that FOXP3 did not directly affect hypothalamic function (Fig. 3, A and C).

Hypothalamic Gnrh *Expression in* Foxp3$^{sf/Y}$ *Mice*

LH production by the pituitary is dependent on GnRH from the hypothalamus. To determine if *Gnrh* expression in the hypothalamus is decreased, we collected hypothalami from wild-type and scurfy mice at 6–9 wk of age and performed real-time RT-PCR to measure *Gnrh* mRNA levels. We found that *Gnrh* levels were not significantly decreased in scurfy mice as compared to wild-type male littermates (Fig. 5A). However, this did not rule out the possibility of a hypothalamic defect. We did detect a significant decrease in pituitary expression of *Gnrhr*, suggesting that the ability of the pituitary gland to respond to GnRH signaling was impaired (Fig. 5B).

Treatment with GnRH Receptor Agonist

We next sought to determine if we could stimulate gonadotropin production in scurfy mice by activating GnRH receptor signaling with pulsatile GnRH analog treatments. *Foxp3*$^{sf/Y}$ mice and wild-type male littermates at 6 wk of age were treated with D-Ala-6-GnRH every 2 h for 48 h. Pituitaries were collected 2 h after the last injection and *Fshb* and *Lhb* mRNA levels were measured. We found that pulsatile GnRH analog treatments significantly increased *Fshb* expression in wild-type males (Fig. 5C). Although there was a trend toward increased *Lhb* expression, the difference was not significant. This could be because the wild-type animals had an intact hypothalamic-pituitary-gonadal axis, and therefore *Lhb* expression may already have been maximally stimulated. In *Foxp3*$^{sf/Y}$ male mice, neither *Fshb* nor *Lhb* expression was significantly increased with D-Ala-6-GnRH treatment (Fig. 5, C and D).

DISCUSSION

FOXP3 is essential for normal immune function [13, 34] because of its role in proper development and function of regulatory T cells [11, 35]. Without FOXP3, humans and mice develop severe autoimmunity, characterized by hypothyroidism, diabetes, and failure to thrive [34, 36]. The gene encoding FOXP3 resides on the X chromosome in mice and humans; thus, males are primarily affected by loss of FOXP3 [13, 19, 34, 35]. Previous reports have indicated that mice with a mutation in the *Foxp3* gene, *Foxp3*$^{sf/Y}$, are infertile [14, 15, 37]. Sharma et al. demonstrated that submandibular gland development, which is sexually dimorphic and dependent on testosterone in males, is inhibited in *Foxp3*$^{sf/Y}$ males, suggesting that they have low testosterone levels, although

testosterone levels have never been directly measured [37]. We find that *Foxp3*$^{sf/Y}$ mice have severely reduced *Lhb* and *Fshb* expression, which can explain the hypogonadism seen in these mice and would contribute to low testosterone.

Several systems are out of balance in *Foxp3*$^{sf/Y}$ mice in consequence of their autoimmunity. Pancreatic β cells are destroyed by immune cells, resulting in type 1 diabetes due to loss of insulin production [33, 34]. Pituitary-specific insulin receptor knockout female mice do have decreased *Lhb* levels as compared to wild-type littermates in the context of obesity [38]. When mice were not obese, no differences in Lhb levels were observed; however, the effects of eliminating pituitary insulin signaling in male mice has not been addressed [38]. Humans with mutations in *FOXP3* exhibit hypothyroidism due to destruction of the thyroid gland [39]. Although, to our knowledge, the thyroid hormone status of *Foxp3*$^{sf/Y}$ mice has not been examined, loss of thyroid hormone could negatively affect gonadotroph function [40]. Finally, *Foxp3*$^{sf/Y}$ mice have high cytokine levels because of the decreased action of regulatory T cells [41]. Cytokines have been shown to inhibit gonadotropin production [42–44]. The loss of insulin, loss of thyroid hormone, and increase in cytokine levels could act individually or in combination to inhibit gonadotroph function.

LH, FSH, and TSH are all dimeric hormones of the glycoprotein hormone family. We find that expression of *Lhb, Fshb*, and *Cga* is reduced in *Foxp3*$^{sf/Y}$ mice as compared to wild-type male littermates, whereas expression of *Tshb* is increased. Immunohistochemistry for TSHβ shows no obvious difference in the number of thyrotrope cells (data not shown). Immunohistochemistry for αGSU does not detect normal levels of staining in any pituitary cells, demonstrating that αGSU is reduced in thyrotropes as well as gonadotrophs. This suggests that *Foxp3*$^{sf/Y}$ mice have reduced levels of biologically active TSH, which could lead to hypothyroidism. Thus, the increased expression levels of *Tshb* may be due to lack of negative feedback, which is ultimately caused by reduced αGSU production in the pituitary.

The *hpg* mice, which have a mutation in the *Gnrh* gene, are infertile because of hypogonadotropic hypogonadism. Treatment of adult *hpg* mice with pulsatile GnRH increased pituitary LH content, but did not increase plasma LH to detectable levels [45]. Hamernik et al. demonstrated that 2-h pulses of GnRH stimulated transcription of *Lhb* in ewes after hypothalamic-pituitary disconnect [46]. Spratt and Crowley found that pituitary and gonadal responsiveness to exogenous GnRH was reduced in men who were deficient in GnRH. Treatment with pulsatile GnRH for a period of 6 mo enhanced pituitary and gonadal responsiveness to physiological doses of exogenous GnRH, suggesting that the pituitary gland and gonads require hypothalamic stimulation in order to mature before they can respond to GnRH appropriately [47]. We find that treating 6-wk-old mutant animals with the GnRH agonist D-ala-6-GnRH does not significantly increase *Lhb* or *Fshb* expression. Although we cannot rule out the possibility that treating *Foxp3*$^{sf/Y}$ mice with GnRH for a longer period of time or at a different pulse frequency may have some effect, failure of the GnRH agonist to stimulate *Lhb* and *Fshb* expression suggests that the gonadotroph cells are not capable of responding normally to GnRH stimulation.

We have marked gonadotroph cells by performing immunohistochemistry for steroidogenic factor-1 (Fig. 4). We do not observe an obvious difference in gonadotroph cell number or expression of the gene encoding SF1 (*Nr5a1*), suggesting that the decrease in gonadotropin production is at the level of *Fshb* and *Lhb* expression rather than gonadotroph cell number. Pituitary from *Foxp3*$^{sf/Y}$ mice does exhibit a decrease in expression of GnRH receptor, suggesting a decrease in gonadotroph responsiveness to GnRH. Consistent with this, *Fshb, Lhb, Cga,* and *Gnrhr* are all GnRH-responsive genes [4, 46, 48–51]. However, this does not rule out the possibility that other factors, such as low insulin or high cytokines, are contributing to diminished gonadotroph function.

When the immune and endocrine systems become unbalanced, for example, cases of increased immune function such as autoimmunity or decreased endocrine function like pituitary hormone deficiency, neither system functions properly. For example, many autoimmune diseases result in subfertility in males and females [52, 53]. Studies of mink with spontaneous autoimmune orchitis find that these animals have high levels of anti-sperm antibodies, high levels of tumor necrosis factor (TNF) α and interleukin (IL) 6, and low levels of testosterone [53]. Pituitary hormone levels were not analyzed in these animals.

The balance between the immune and reproductive systems is maintained, in part, because these systems are modulated by many of the same factors, including GnRH, estradiol, and cytokines. In fact, *Gnrh* is expressed in the thymus as well as in the hypothalamus and is important for thymic maturation and differentiation [54]. Estrogens can promote or inhibit inflammation [55, 56]. Several cytokines, including IL1, IL2, IL6, IFNG, and TNF, are produced in the hypothalamus and/or pituitary [43]. GH, PRL, and LH are also produced in the thymus. In addition, receptors for several cytokines and hormones are expressed in both the thymus and the hypothalamic/pituitary axis [43]. There are many examples that demonstrate cytokine regulation of reproductive function; for example, IL2 has been shown to stimulate *Pomc* expression and inhibit LH, FSH, and GH release [43]. Central administration of IL1 drastically inhibits GnRH production; however, systemic IL1 appears to have little effect [42]. TNF has been shown to inhibit release of GH, LH, and PRL [44]. Thus, one can see how an imbalance in the immune system, such as elevated cytokines, can have a huge effect on reproductive function.

We do not detect *Foxp3* expression above background in the pituitary or hypothalamus, suggesting that the loss of gonadotropin production in *Foxp3*$^{sf/Y}$ mice is not due to a primary defect of the hypothalamus or pituitary, but rather is secondary to loss of functional FOXP3 protein in some other tissue. Gonadotroph cells are readily detected by immunohistochemistry for SF1, suggesting that they have not been destroyed by the immune system. One possibility is that *Foxp3* is required for maintaining the balance between the immune system and reproductive system and loss of *Foxp3* disrupts this balance, resulting in infertility. Interestingly, another forkhead factor that is important for regulating immune function is FOXN1. A spontaneously occurring recessive mutation in the murine *Foxn1* gene, referred to as nude (*nu*), causes the mice (*Foxn1*$^{nu/nu}$) to be immunocompromised and athymic. Congenitally athymic *Foxn1*$^{nu/nu}$ female mice are subfertile, exhibiting low gonadotropin levels. Although *Foxn1*$^{nu/nu}$ male mice are fertile, they too exhibit significantly reduced gonadotropin levels [57]. Injecting wild-type mice with antibodies against thymulin, a thymic hormone, can recapitulate the reduction in gonadotropin levels seen in athymic animals [58].

Breeding the *sf* mutation onto a nude (*Foxn1*$^{nu/nu}$) mouse background (*Foxp3*$^{sf/Y}$; *Foxn1*$^{nu/nu}$) rescues both the *Foxp3*$^{sf/Y}$ autoimmunity and infertility; however, *Foxp3*$^{sf/Y}$; *Foxn1*$^{nu/nu}$ mice retain the *Foxn1*$^{nu/nu}$ immunodeficiency [15]. In other words, breeding the *sf* mutation on a nude background rescues the scurfy phenotype, including infertility, but does not rescue

the nude phenotype. The fact that fertility in *Foxp3*$^{sf/Y}$ males is rescued when the mice are congenitally athymic (by breeding them to nude mice) suggests that the infertility in *Foxp3*$^{sf/Y}$ mice is secondary to their autoimmunity, which is dependent on the presence of the thymus. Lack of *Foxp3* expression in the pituitary and hypothalamus, together with the fact that fertility is rescued by loss of thymic function, suggests that infertility in *Foxp3*$^{sf/Y}$ mice is secondary to their autoimmunity.

Together, these data demonstrate that FOXP3 is required for fertility. We find that LH production is suppressed in *Foxp3*$^{sf/Y}$ mice even though *Foxp3* is not expressed in the pituitary, suggesting that the loss of LH production is secondary to loss of FOXP3 in another tissue. Godfrey et al. demonstrated that breeding *Foxp3*$^{sf/Y}$ mice onto a *Foxn1*$^{nu/nu}$ background rescued their autoimmunity and infertility, suggesting that their immune defects are the primary cause of their infertility [15]. Thus, in the absence of FOXP3, Treg cells fail to develop and immune activity escalates, ultimately resulting in suppression of the endocrine system. Although *Foxp3* is not expressed in the pituitary, we find that it is essential for normal pituitary function.

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