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The Forkhead Transcription Factor, FOXP3: A Critical Role in Male Fertility in Mice[1]

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ABSTRACT

Fertility is dependent on the hypothalamic-pituitary-gonadal axis. Each component of this axis is essential for normal reproductive function. Mice with a mutation in the forkhead transcription factor gene, *Foxp3*, exhibit autoimmunity and infertility. We have previously shown that *Foxp3* mutant mice have significantly reduced expression of pituitary gonadotropins. To address the role of *Foxp3* in gonadal function, we examined the gonadal phenotype of these mice. *Foxp3* mutant mice have significantly reduced seminal vesicle and testis weights compared with $Foxp3^{+/Y}$ littermates. Spermatogenesis in *Foxp3* mutant males is arrested prior to spermatid elongation. Activation of luteinizing hormone signaling in *Foxp3* mutant mice by treatment with human chorionic gonadotropin significantly increases seminal vesicle and testis weights as well as testicular testosterone content and seminiferous tubule diameter. Interestingly, human chorionic gonadotropin treatments rescue spermatogenesis in *Foxp3* mutant males, suggesting that their gonadal phenotype is due primarily to a loss of pituitary gonadotropin stimulation rather than an intrinsic gonadal defect.

fertility, forkhead, FOXP3, gonadotropin, pituitary, spermatogenesis, transcription factor

INTRODUCTION

Central to reproductive function is the hypothalamic-pituitary-gonadal axis, in which hypothalamic gonadotropin-releasing hormone (GnRH) binds to specific receptors on the surface of gonadotroph cells to stimulate synthesis of the gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Pituitary gonadotropins are heterodimers consisting of a common α subunit and unique β subunits, which confer their specialized functions. Luteinizing hormone binds to receptors on Leydig cells to stimulate testosterone production, which is essential for spermatogenesis to proceed [1, 2]. Follicle-stimulating hormone regulates Sertoli cell number and stimulates maintenance of spermatogenesis [3–5].

Balance between immune function and endocrine function is essential for normal homeostasis. When these systems become unbalanced—for example, in cases of increased immune function, such as autoimmunity, or decreased endocrine function, such as pituitary hormone deficiency—neither system functions properly. For this reason, many autoimmune diseases result in subfertility in males and females [6, 7].

The forkhead factor, FOXP3, plays important roles in the differentiation and function of regulatory T cells [8]. 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 [9]. Death often occurs during the first years of life [9].

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 [10]. Interestingly, affected males ($Foxp3^{sf/Y}$) have small testes, are sterile, and appear hypogonadal; however, no hormonal studies have been done [11, 12]. Recently, we found that pituitary expression of *Lhb*, *Fshb*, and *Cga* is significantly reduced in $Foxp3^{sf/Y}$ male mice [13]. In the following studies we characterize the gonadal phenotype in $Foxp3^{sf/Y}$ male mice to determine whether any intrinsic testicular defects are present.

MATERIALS AND METHODS

Mice

Foxp3 mutant mice were purchased from the Jackson Laboratory and maintained on a C57BL/6J background. $Foxp3^{sf/Y}$ females were mated to $Foxp3^{+/Y}$ males to obtain $Foxp3^{sf/Y}$ male offspring. $Foxp3^{sf/Y}$ male mice were left with dams to increase survival time. Mice were maintained on a 12L:12D cycle. To genotype mice, a Custom TaqMan SNP Genotyping Assay (Applied Biosystems) was used according to the manufacturer's instructions. Male mice were used for all studies.

All procedures using mice were approved by the Southern Illinois University Animal Care and Use Committee. All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

Histology and Immunohistochemistry

Each testis was incubated in Bouin fixative overnight at room temperature. Each testis was washed in 50% ethanol, then in 80% ethanol on ice before embedding. Serial sections were cut to 5 μm and stained with periodic acid-Schiff-hematoxylin (PASH).

Immunohistochemistry was performed by incubating tissue sections with specific antibodies that recognize 3 hydroxysteroid dehydrogenase (HSD3B1; provided by the late Dr. Anita Payne, Stanford University) at a dilution of 1:800 for 1 h at room temperature. Biotinylated secondary antibody was applied for 10 min, followed by Vectastain Elite ABC reagent (Vector Laboratories). Diaminobenzidine was added to visualize HSD3B1, and tissue sections were counterstained with hematoxylin.

Human Chorionic Gonadotropin Treatment

$Foxp3^{sf/Y}$ mice and $Foxp3^{+/Y}$ littermates were injected with 5 IU of human chorionic gonadotropin (hCG) starting at Postnatal Day 28 (P28) or P14.

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Animals were injected with hCG dissolved in NaCl (0.15 M) or with vehicle every 48 h for the duration of the treatment period. At P42, seminal vesicles and testes were collected and weighed. Testis and seminal vesicle weights were adjusted for total body weight. For each individual, one testis was snap frozen for testosterone measurement, and one was incubated in Bouin fixative and stained with PASH as described above. Each treatment group contained at least four animals.

To count elongated spermatids, 1 testis was analyzed per individual, 4 sections from each PASH-stained testis were analyzed, and 17 seminiferous tubules were counted per section. Counts for each testis were averaged together to obtain one value per individual. Individuals in each treatment group were averaged together to calculate the average number of elongated spermatids per treatment group and to determine the standard error around the mean. Four individuals were analyzed per treatment group.

Seminiferous tubule diameter was measured for the same tubules that were analyzed for elongated spermatid number. Seminiferous tubule diameter was measured using QCapture Pro software (version 6.0; QImaging). Measurements are expressed in micrometers. Measurements for each testis were averaged together to get one value per individual. Individuals in each treatment group were averaged together to calculate the average seminiferous tubule diameter per treatment group and to determine the standard error around the mean. Four individuals were analyzed per treatment group.

Testicular Testosterone Assays

Each testis was dissected, weighed, and snap frozen in liquid nitrogen. Testis tissue was homogenized, extracted with diethyl ether, and measured using the Parameter Testosterone Assay (R&D Systems) as per manufacturer instructions. At least four animals were included in each group.

RT-PCR

Pituitary, thymus, and testis tissues were dissected from mice and stored in RNAlater (Ambion Inc.) at −20°C. Total RNA was extracted and DNase treated using the RNAqueous-Micro Kit (AM1931; Ambion by Life Technologies,) per the manufacturer's instructions, and RNA concentrations were determined by spectrophotometry. The RT-PCR procedure employed the TaqMan RNA-to-C_T 1-Step Kit (4392938; Applied Biosystems by Life Technologies Inc.) according to the manufacturer's directions and CFX96 Real Time System (Bio-Rad). Expression levels for *Foxp3* (TaqMan probe Mm00475156_m1; Applied Biosystems by Life Technologies) *Lhb* (Mm00656868_g1), *Fshb* (Mm00433361_m1), and *Foxo1* (Mm00490672_m1) were determined using β-actin (*Actb*; 4352933E) as the endogenous control (all TaqMan probes from Applied Biosystems Inc.). Fifty nanograms of cDNA was used in 20-μl reaction volumes in triplicate, and no-template and no-reverse transcriptase controls were used to ensure the absence of contamination and efficacy of the DNase treatment, respectively. At least four individuals were included in each group for all real-time experiments. Amplification was achieved by the following protocol: 48°C for 15 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. *Foxp3* and correlating *Actb* reactions were visualized on a 1% agarose gel. Relative quantification analysis was performed using the comparative C_T method $2^{-\Delta\Delta CT}$. The values for $\Delta\Delta C_T$ were calculated by subtracting the average ΔC_T of wild-type controls from the ΔC_T for each sample.

Thyroid Treatment

Purina Test Diets provided thyroid hormone-enriched mouse chow consisting of special pelleted AIN-76A diet with thyroid gland powder added at a concentration of 25 mg/kg chow. $Foxp3^{+/sf}$ female mice were mated to C57BL6/J males. The date the copulatory plug was detected was considered to be Embryonic Day 0.5. Pregnant $Foxp3^{+/sf}$ female mice were fed either thyroid chow or control chow ad libitum starting at Embryonic Day 16.5. $Foxp3^{sf/Y}$ pups were housed with dams throughout the treatment period. At 6 wk of age pituitary tissue was collected. At least five animals were included in each treatment group.

Statistical Analysis

Data are expressed as a mean ± SEM. Data were analyzed by Student *t*-test to determine significant difference between $Foxp3^{+/Y}$ and $Foxp3^{sf/Y}$ mice or between different treatment groups (Microsoft Excel 2004 for Mac version 11.6.6). *P* values of less than 0.05 were considered statistically significant (*); *P* values less than 0.01 were considered very significant (**).

RESULTS

Gonadal Phenotype of Foxp3$^{sf/Y}$ Male Mice

Studies of $Foxp3^{sf/Y}$ male mice have suggested that they are hypogonadal and sterile [11, 12]. Consistent with this, $Foxp3^{sf/Y}$ mice have very low gonadotropin expression [13], suggesting that their hypogonadism may be hypogonadotropic in nature.

To determine whether *Foxp3* is expressed in the testis, RT-PCR was performed using specific primers for *Foxp3* and *Actb*. *Foxp3* is expressed in thymus; thus, thymus was used as a positive control for the *Foxp3* primers. *Foxp3* primers amplified a band from thymus and from testis, suggesting that *Foxp3* transcript is present in testis (Fig. 1A). We next performed immunohistochemistry to determine which cell types in the testis express FOXP3. Although FOXP3 protein was detected in spleen, none was detected in testis (Supplemental Fig. S1, available online at www.biolreprod.org). This suggests that although *Foxp3* transcript is produced in the testis, FOXP3 protein is not.

To more carefully characterize the testicular phenotype and determine whether intrinsic testicular defects occur in the absence of *Foxp3*, the anatomy of $Foxp3^{sf/Y}$ reproductive tracts was observed (Fig. 1B). Seminal vesicles and testes from $Foxp3^{sf/Y}$ mice were much smaller than those of $Foxp3^{+/Y}$ littermates. The epididymis of $Foxp3^{sf/Y}$ mice was hypoplastic. Consistent with previous reports [14], overall body size of $Foxp3^{sf/Y}$ males was significantly reduced (Fig. 1C). Testicular size and seminal vesicle size were both significantly reduced, even when adjusted for body size (Fig. 1, D and E). Testicular testosterone content was significantly reduced in $Foxp3^{sf/Y}$ mice compared with $Foxp3^{+/Y}$ littermates (Fig. 1F).

In light of their decreased testis and seminal vesicle weights, we hypothesized that $Foxp3^{sf/Y}$ mice would have abnormal testicular morphology. To assess testicular morphology, a PASH stain was performed on testis sections from $Foxp3^{sf/Y}$ mice and $Foxp3^{+/Y}$ littermates. These studies revealed that spermatogenesis is disrupted in $Foxp3^{sf/Y}$ mice (Fig. 2, A–C). Although spermatogonia and spermatocytes were present in $Foxp3^{sf/Y}$ testis, elongated spermatids were not observed. Because testicular testosterone levels were reduced in $Foxp3^{sf/Y}$ mice, we next sought to determine whether Leydig cells were present in normal numbers. Leydig cells were labeled using antibodies that specifically recognize HSD3B1. Although Leydig cells were present in $Foxp3^{sf/Y}$ testis, the size of the clusters was apparently reduced (Fig. 2, D–F).

Activation of LH Receptor Signaling in Foxp3$^{sf/Y}$ Mice

Previously, we found that pituitary gonadotropin production is significantly reduced in $Foxp3^{sf/Y}$ mice [13]. To determine whether the arrest in spermatogenesis is due entirely to loss of gonadotropin stimulation, these mice were treated with hCG to stimulate the LH signaling pathway. $Foxp3^{sf/Y}$ mice and $Foxp3^{+/Y}$ littermates were injected with 5 IU of hCG every other day starting at P28 and continuing until P42, when tissues were collected (Fig. 3A). Treatment with hCG rescued the testicular phenotype by increasing seminiferous tubule size, but it did not rescue spermatogenesis (Fig. 3, B–G). Treatment with hCG increased testis weight, seminal vesicle weight, and testicular testosterone content in $Foxp3^{sf/Y}$ mice (Fig. 3, E–G). These data suggest that treating $Foxp3^{sfY}$ mice with hCG for 14 days begins to rescue their testicular phenotype.

To determine whether longer treatment with hCG could rescue the testicular phenotype of $Foxp3^{sfY}$ mice more completely, mice were treated with 5 IU of hCG starting at

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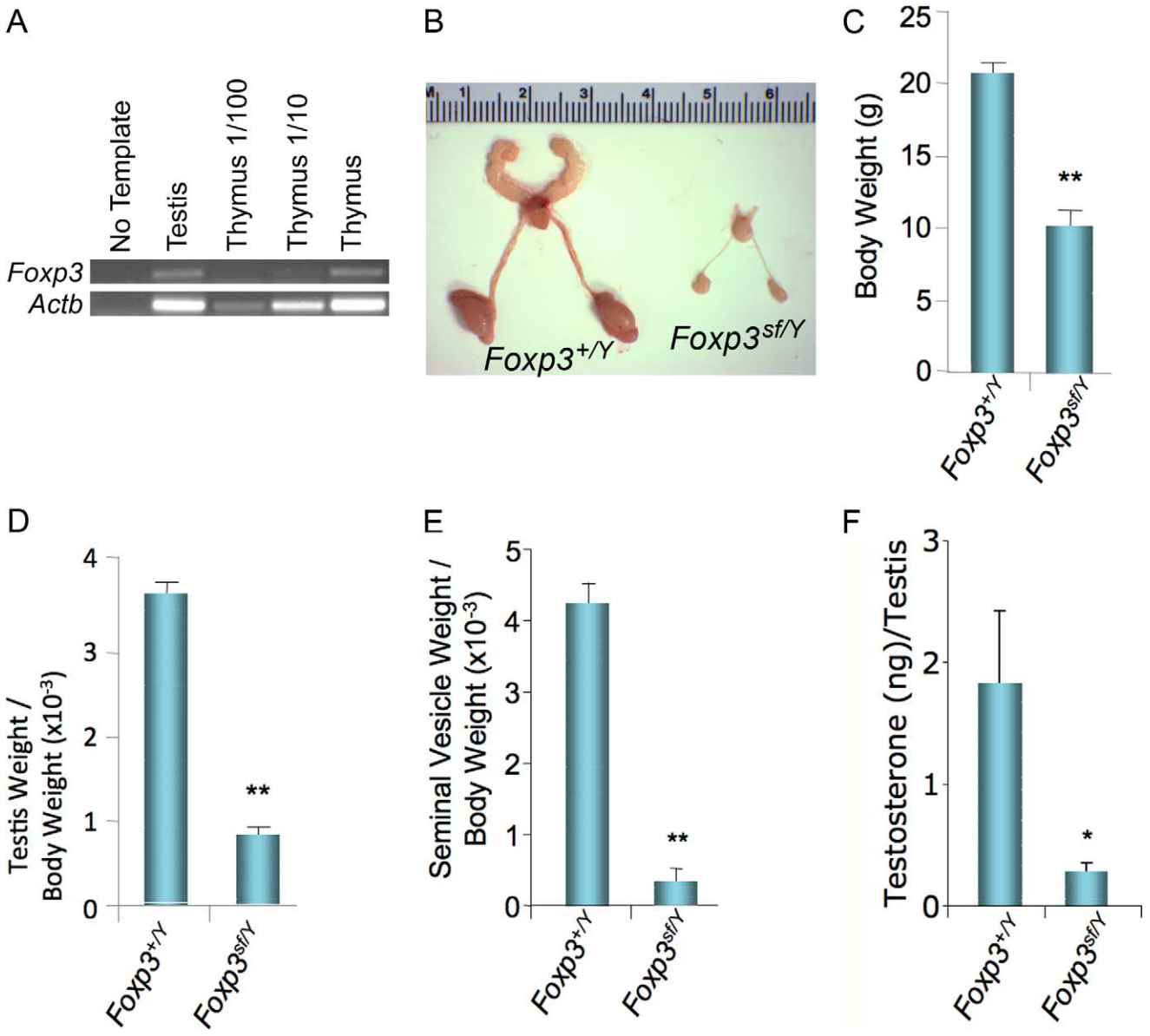

FIG. 1. **A**) *Foxp3* mRNA is detected in the testis at 6 wk of age. **B**) Reproductive tracts from $Foxp3^{+/Y}$ and $Foxp3^{sf/Y}$ mice at 6 wk of age. $Foxp3^{sf/Y}$ mice exhibit a reduction in body weight (**C**; 20.55 ± 0.52 vs. 9.90 ± 1.06 g), testis weight/body weight (**D**; $3.70 \times 10^{-3} \pm 0.09 \times 10^{-3}$ vs. $0.82 \times 10^{-3} \pm 0.09 \times 10^{-3}$), and seminal vesicle weight/body weight (**E**; $4.26 \times 10^{-3} \pm 0.25 \times 10^{-3}$ vs. $0.31 \times 10^{-3} \pm 0.17 \times 10^{-3}$) at 6 wk of age. **F**) Testicular testosterone content is significantly reduced in $Foxp3^{sf/Y}$ mice compared with $Foxp3^{+/Y}$ littermates (1.97 ± 0.56 vs. 0.27 ± 0.06 ng per testis). Data are expressed as mean ± SEM of at least seven animals per genotype. The data were analyzed by Student *t*-test to determine significant difference between $Foxp3^{sf/Y}$ and $Foxp3^{+/Y}$ littermates ($^*P < 0.05$, $^{**}P < 0.01$).

P14 and continuing until P42 (Fig. 4A). The longer treatment regimen significantly increased testis weight, seminal vesicle weight, the number of elongated spermatids, and seminiferous tubule diameter (Fig. 4, B–E). Elongated spermatids were present in testis from all $Foxp3^{sfY}$ mice treated with 5 IU of hCG for 4 wk. Histological analysis of testis sections revealed that testis from $Foxp3^{sfY}$ mice is very similar to that of their $Foxp3^{+/Y}$ littermates (Fig. 4, F–H). Thus, spermatogenesis in $Foxp3^{sfY}$ mice is rescued by 28 days of hCG treatment. Considering the entire process of spermatogenesis is approximately 35 days in mice [15], it is likely that an even longer treatment would cause a more complete rescue. These data indicate that the testicular phenotype is largely due to loss of gonadotropin stimulation.

Hypothyroidism and Infertility

Humans with *FOXP3* mutation are hypothyroid because of immune destruction of the thyroid gland [16]. Thyroid-stimulating hormone levels are a very sensitive indicator of hypothyroidism [17]. Previously, we found that *Tshb* expression is elevated in $Foxp3^{sf/Y}$ mice, suggesting that like many humans with IPEX, $Foxp3^{sf/Y}$ mice also exhibit hypothyroidism [13]. Evidence suggests hypothyroidism in males can cause abnormal gonadotropin levels [18, 19]. To determine whether treatment with thyroid hormone could rescue gonadotropin levels in $Foxp3^{sf/Y}$ mice, pregnant $Foxp3^{sf/+}$ dams were fed chow containing thyroid powder or a control chow beginning at Embryonic Day 16.5 (Fig. 5A). $Foxp3^{sf/Y}$ pups remained with their dams and continued to receive their respective diets until 6 wk of age, when tissues were collected. $Foxp3^{sf/Y}$ mice

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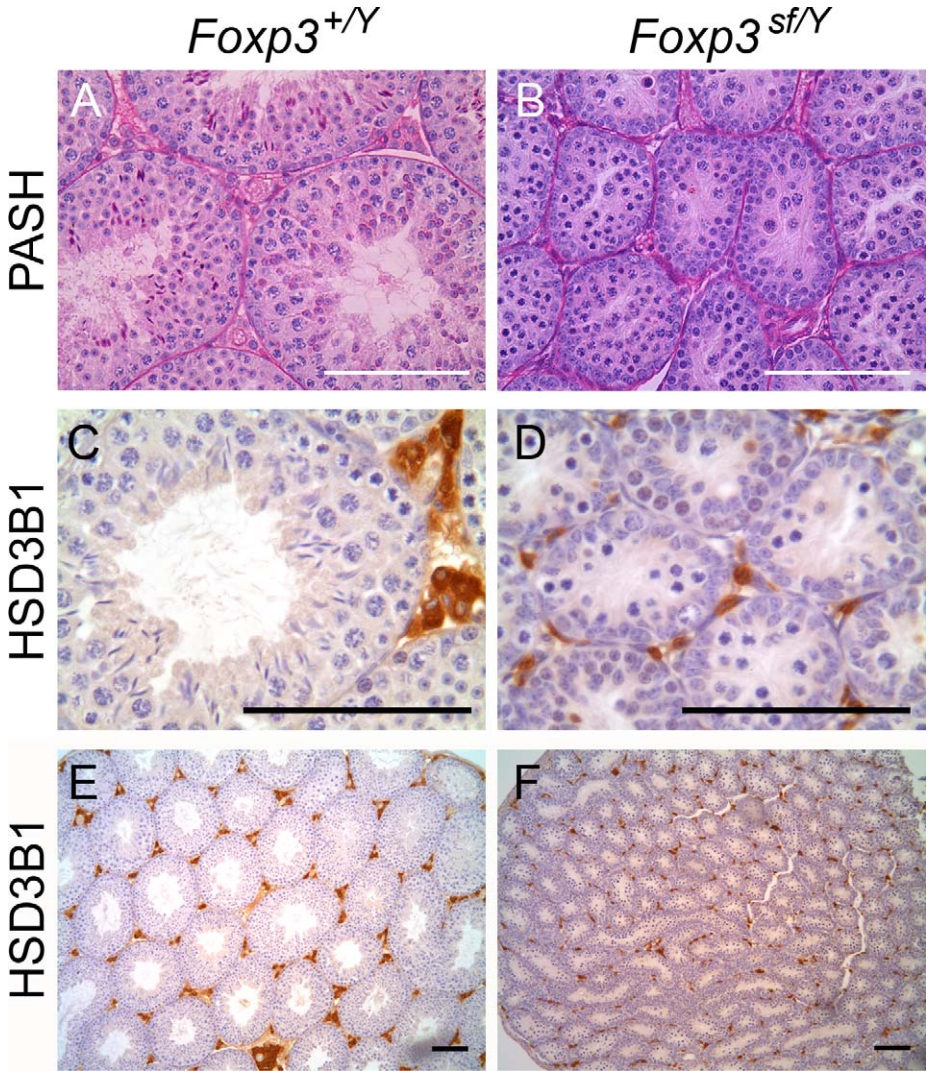

FIG. 2. Testis sections from *Foxp3*$^{+/Y}$ (**A**) and *Foxp3*$^{sf/Y}$ (**B**) mice were stained with PASH, revealing that seminiferous tubule diameter is reduced in *Foxp3*$^{sf/Y}$ mice and spermatogenesis is arrested. Immunohistochemistry for HSD3B1 was used to label Leydig cells in testis from *Foxp3*$^{+/Y}$ (**C** and **E**) and *Foxp3*$^{sf/Y}$ (**D** and **F**) mice. *Foxp3*$^{sf/Y}$ mice exhibit smaller clusters of Leydig cells than their wild-type littermates. Four animals were analyzed per genotype. Original magnifications ×400 (**A** and **B**), ×630 (**C** and **D**), and ×100 (**E** and **F**); bar = 100 μm.

that were fed thyroid chow exhibited a very significant reduction in *Tshb* expression, consistent with the reversal of hypothyroidism (Fig. 5B). No significant change in *Fshb* or *Lhb* expression levels occurred when *Foxp3*$^{sf/Y}$ mice were fed thyroid chow, indicating that hypothyroidism is not the cause of the infertility seen in *Foxp3*$^{sf/Y}$ mice (Fig. 5, C and D). Thyroid treatment did not affect body weight, seminal vesicle weight, or testis weight, nor did it rescue spermatogenesis (data not shown).

DISCUSSION

Foxp3$^{sf/Y}$ *Male Mice Are Hypogonadal and Infertile*

Foxp3 mutant males are infertile and exhibit reduced gonadotropin expression [12, 13]. Although *Foxp3* transcript is present in the testis, FOXP3 protein is not detected. The testicular phenotype of *Foxp3*$^{sf/Y}$ mice is very similar to that of mice lacking gonadotropins. *Hpg* mice are gonadotropin deficient because of a spontaneous mutation in the *Gnrh* gene [20, 21]. Treatment of *hpg* mice with hCG for 6 wk rescues spermatogenesis and testis size, but these parameters do not reach normal levels, suggesting that other factors, such as FSH, are important for quantitative normalization of spermatogenesis [22]. Mice with deletions of the genes encoding for LHB (*Lhb*) or the receptor for LH (*Lhcgr*) are hypogonadal and infertile, with reduced testosterone levels, resulting in spermatogenesis being arrested at the round spermatid stage [23–25]. The similarity between *Foxp3*$^{sf/Y}$ mice and *Lhb* and *Lhcgr* null mice, combined with the rescue of spermatogenesis in *Foxp3*$^{sf/Y}$ mice by activating LH signaling, suggests that the gonadal phenotype in *Foxp3*$^{sf/Y}$ mice is due primarily to a lack of pituitary LH stimulation. This does not rule out the possibility that the immune system is directly inhibiting gonadal function and that treatment with hCG causes hyperstimulation of the testis, overcoming immune suppression of testis.

When bred onto a nude mouse background, which eliminates their autoimmunity, *Foxp3* mutant males become fertile, suggesting that their infertility is secondary to autoimmunity [12]. Nude mice have an autosomal recessive mutation of *Foxn1*, causing them to be athymic and immunosuppressed [26]. Godfrey et al. [14] bred scurfy mice onto a nude mouse background to create *Foxp3*$^{sf/Y}$,*Foxn1*$^{nu/nu}$ mice. These mice no longer exhibited autoimmunity. They did exhibit immunosuppression consistent with that observed in

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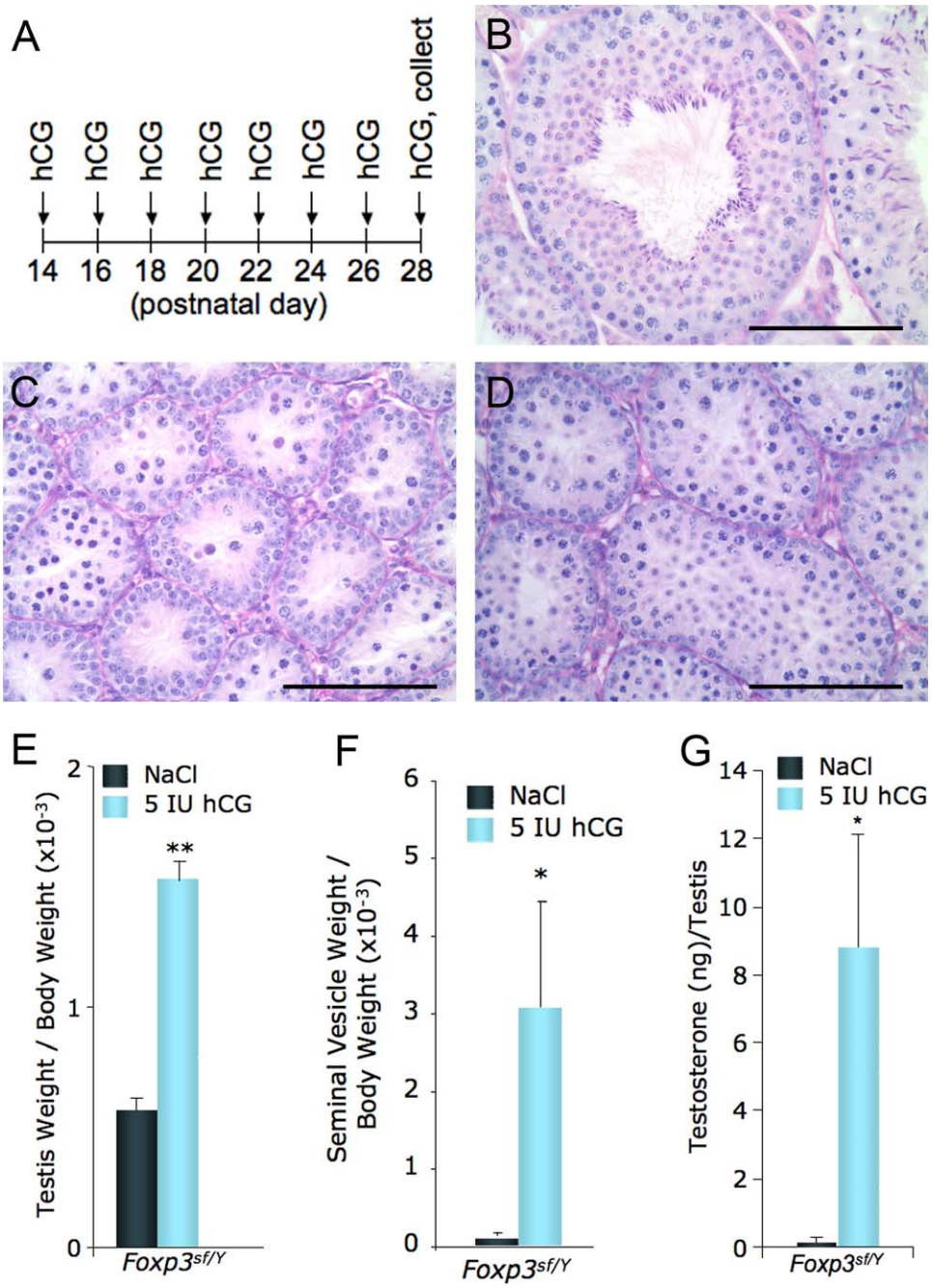

FIG. 3. **A**) *Foxp3*$^{sf/Y}$ mice were treated with 5 IU of hCG (n = 5) or vehicle (n = 5) every other day for 14 days. Testis sections from *Foxp3*$^{+/Y}$ mouse treated with vehicle (**B**), *Foxp3*$^{sf/Y}$ mouse treated with vehicle (**C**), and *Foxp3*$^{sf/Y}$ mouse treated with 5 IU of hCG (**D**) are shown. Original magnification ×400; bar = 100 μm. **E**) Average testis weight/body weight of *Foxp3*$^{+/Y}$ mice is $3.63 \times 10^{-3} \pm 0.08 \times 10^{-3}$ (data not shown). Testis weights are increased significantly in *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG ($1.55 \times 10^{-3} \pm 0.05 \times 10^{-3}$) compared with *Foxp3*$^{sf/Y}$ mice treated with vehicle ($0.56 \times 10^{-3} \pm 0.06 \times 10^{-3}$). **F**) Average seminal vesicle weight/body weight of *Foxp3*$^{+/Y}$ mice is $2.98 \times 10^{-3} \pm 0.34 \times 10^{-3}$ (data not shown). Seminal vesicle weights are significantly increased with 5 IU of hCG ($3.06 \times 10^{-3} \pm 0.14 \times 10^{-3}$) compared with *Foxp3*$^{sf/Y}$ mice treated with vehicle ($0.10 \times 10^{-3} \pm 0.02 \times 10^{-3}$). **G**) Average testicular testosterone levels for *Foxp3*$^{+/Y}$ mice treated with vehicle are 7.13 ± 4.12 ng per testis (data not shown). Testicular testosterone levels increased significantly in *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG for 2 wk (8.81 ± 3.27 ng per testis) compared with *Foxp3*$^{sf/Y}$ mice treated with vehicle (0.21 ± 0.03 ng per testis). Data are expressed as mean ± SEM of four animals per group. The data were analyzed by Student *t*-test to determine significant difference between *Foxp3*$^{sf/Y}$ mice treated with hCG or vehicle (*$P < 0.05$, **$P < 0.01$).

Foxn1$^{nu/nu}$ mice. Interestingly, *Foxp3*$^{sf/Y}$,*Foxn1*$^{nu/nu}$ mice were capable of siring progeny. This allowed for generation of *Foxp3*$^{sf/sf}$,*Foxn1*$^{+/nu}$ female mice, which exhibited scurfylike lesions and life spans of <30 days. The microscopic anatomy in the female reproductive tracts was normal [12]. We find that *Foxp3* is not expressed in the adult pituitary gland [13]. This, together with the fact that eliminating autoimmunity in *Foxp3*$^{sf/Y}$ mice by breeding them onto a nude mouse background rescues their fertility, suggests that the reproductive phenotype in *Foxp3*$^{sf/Y}$ mice is secondary due to loss of *Foxp3* in regulatory T cells.

Foxp3$^{sf/Y}$ mice have elevated levels of many cytokines, including interleukin 2 (IL2), IL4, IL5, IL7, IL10, interferon γ (IFNγ), and tumor necrosis factor α (TNFα) [27–29]. There are

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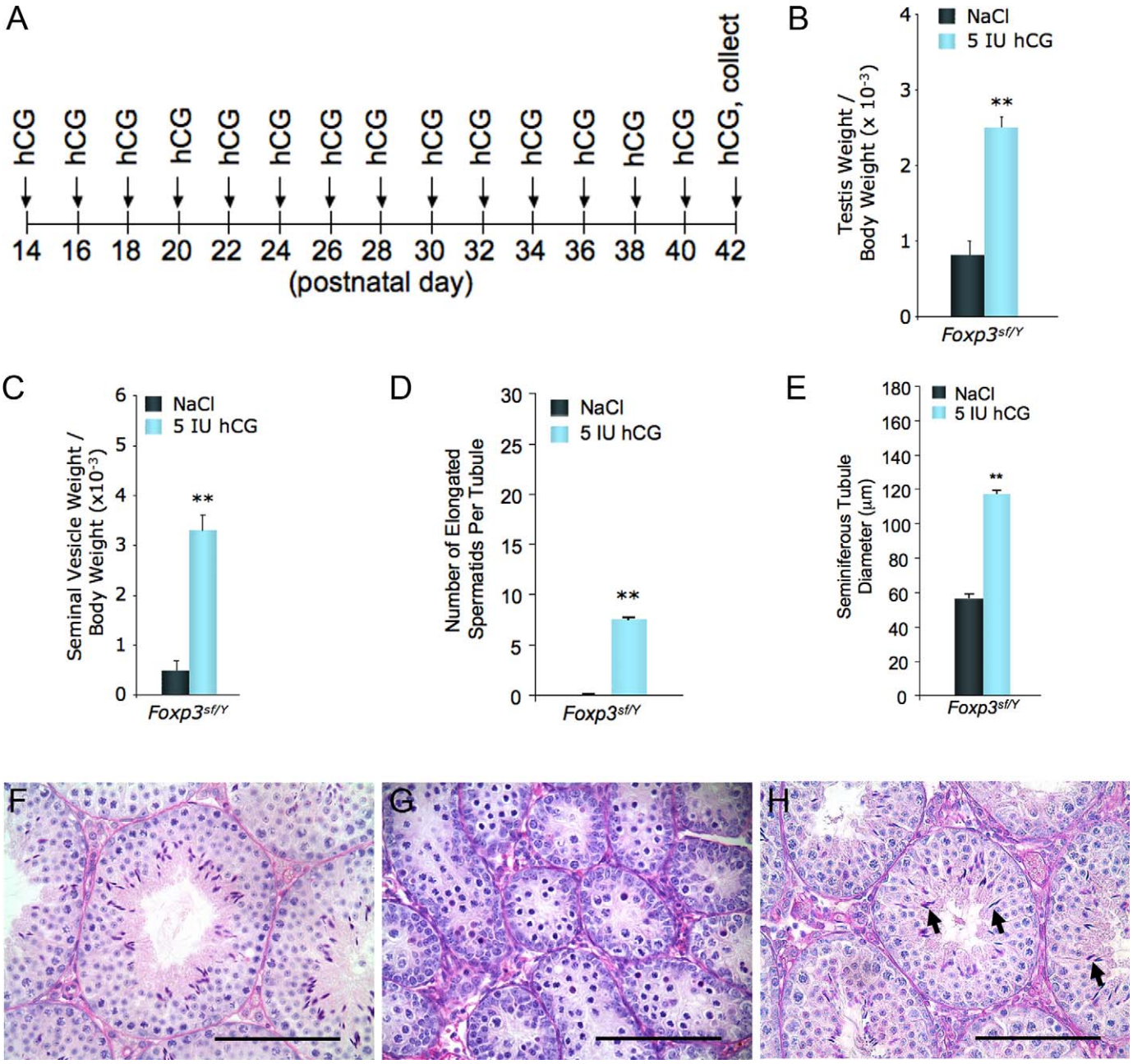

FIG. 4. **A**) *Foxp3*$^{sf/Y}$ mice were treated with 5 IU of hCG (n = 5) or vehicle (n = 4) every other day starting at P14 and continuing to P42. **B**) Average testis weight/body weight for *Foxp3*$^{+/Y}$ mice treated with vehicle is $3.24 \times 10^{-3} \pm 0.50 \times 10^{-3}$ (data not shown). Testis weights increased very significantly in *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG for 4 wk ($2.49 \times 10^{-3} \pm 0.15 \times 10^{-3}$) compared with *Foxp3*$^{sf/Y}$ mice treated with vehicle ($0.82 \times 10^{-3} \pm 0.18 \times 10^{-3}$). **C**) Average seminal vesicle weight/body weight for *Foxp3*$^{+/Y}$ mice treated with vehicle is $4.30 \times 10^{-3} \pm 0.34 \times 10^{-3}$ (data not shown). Seminal vesicle weights increased very significantly in *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG for 28 days ($3.31 \times 10^{-3} \pm 0.63 \times 10^{-3}$) compared with *Foxp3*$^{sf/Y}$ mice treated with vehicle ($0.50 \times 10^{-3} \pm 0.19 \times 10^{-3}$). **D**) The average number of elongated spermatids per cross section of seminiferous tubule is 23 ± 5 for *Foxp3*$^{+/Y}$ mice treated with vehicle, whereas *Foxp3*$^{sf/Y}$ mice treated with vehicle lack elongated spermatids. However, spermatogenesis is rescued in five of five *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG for 28 days, with an average of 7 ± 2 elongated spermatids per cross section of seminiferous tubule. **E**) Average seminiferous tubule diameter of *Foxp3*$^{+/Y}$ mice treated with vehicle is 164 ± 2 μm. Seminiferous tubule diameter was significantly increased in *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG (117 ± 2 μm) compared with *Foxp3*$^{sf/Y}$ mice treated with vehicle (57 ± 3 μm). Data are expressed as mean ± SEM and were analyzed by Student *t*-test to determine significant difference between *Foxp3*$^{sf/Y}$ mice treated with 5 IU of hCG or vehicle (**$P < 0.01$). Testis sections from *Foxp3*$^{+/Y}$ mouse treated with vehicle (**F**), *Foxp3*$^{sf/Y}$ mouse treated with vehicle (**G**), and *Foxp3*$^{sf/Y}$ mouse treated with 5 IU of hCG (**H**) for 28 days are shown. Testis from *Foxp3*$^{sf/Y}$ mice treated with hCG for 28 days contains elongated spermatids (arrows). Original magnification ×400; bar = 100 μm.

many examples of cytokine regulation of reproductive function; for example, IL2 has been shown to stimulate *Pomc* expression and inhibit LH, FSH, and growth hormone release [30]. TNFα has been shown to inhibit release of growth hormone, LH, prolactin, and GnRH [31, 32]. Considering the lack of *Foxp3* expression in hypothalamus and pituitary, it is unlikely that FOXP3 directly effects gonadotropin or *Gnrh* expression [13]. It is possible that the gonadal phenotype observed in *Foxp3*$^{sf/Y}$ mice is due to cytokine inhibition of gonadotropin expression or GnRH release.

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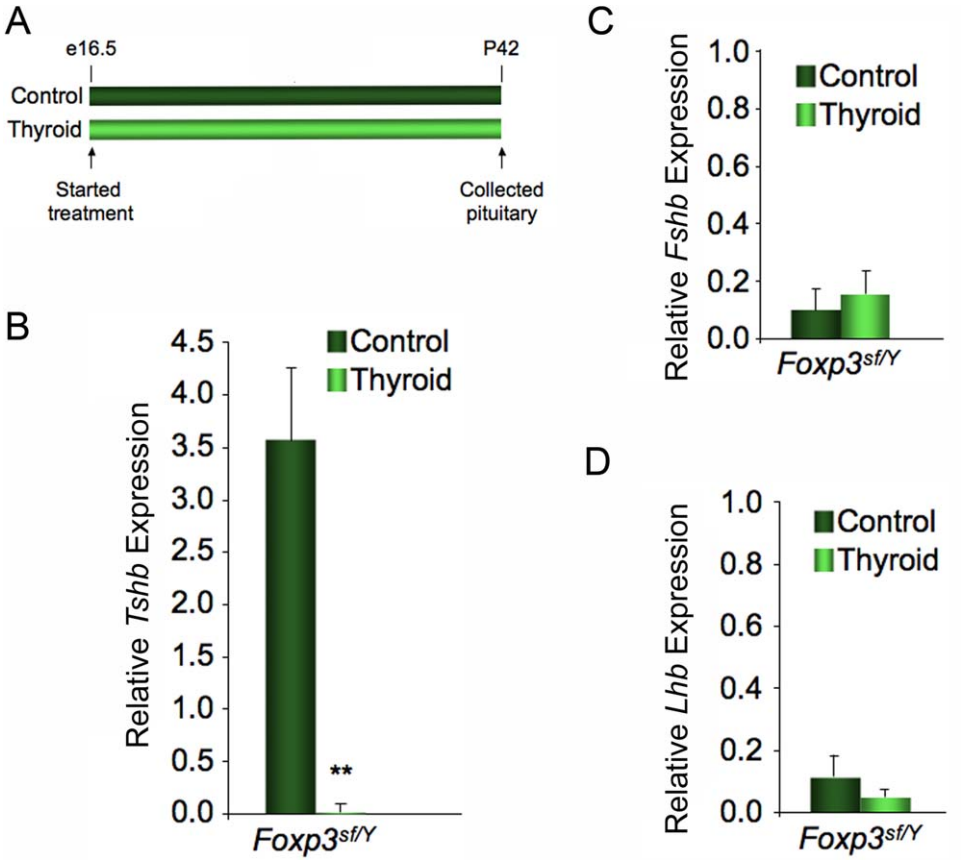

FIG. 5. **A**) Pregnant dams were fed control chow or chow supplemented with thyroid powder beginning at Embryonic Day 16.5. Dams and pups were fed their respective diets until the pups reached 6 wk of age. Real-time RT-PCR was used to determine the relative expression levels of *Tshb* (**B**), *Fshb* (**C**), and *Lhb* (**D**) in pituitary gland tissue of $Foxp3^{sf/Y}$ mice on thyroid versus control chow diets. Thyroid hormone replacement significantly reduced expression of *Tshb* in $Foxp3^{sf/Y}$ mice (0.02) compared with $Foxp3^{sf/Y}$ mice fed control chow (3.57 ± 0.70), but it had no effect on expression of *Fshb* (0.15 ± 0.08) or *Lhb* (0.05 ± 0.03) compared with $Foxp3^{sf/Y}$ mice fed control diet (0.10 ± 0.08 and 0.11 ± 0.07, respectively). Expression level was calculated by the $2^{-\Delta\Delta CT}$ method and represents expression relative to the average ΔC_T of samples from $Foxp3^{+/Y}$ mice fed control diet. Data are expressed as mean ± SEM of four animals per group. The data were analyzed by Student *t*-test to determine significant difference between control and thyroid chow diets (**$P < 0.01$).

Hypothyroidism with Loss of FOXP3

Humans with *FOXP3* mutations often exhibit hypothyroidism due to autoimmune destruction of the thyroid gland [17]. Few studies address thyroid function in $Foxp3^{sf/Y}$ mice. Sharma et al. [33] found no inflammation in pancreas or thyroid. However, Lahl et al. [34] observed immune infiltrate and destruction of the islets in the acini of pancreas in $Foxp3^{sf/Y}$ mice. Unfortunately, they did not examine thyroid tissue from $Foxp3^{sf/Y}$ mice [34]. We find that $Foxp3^{sf/Y}$ mice have elevated *Tshb* expression, which is reversed by thyroid hormone replacement, suggesting that *Foxp3* mutant mice are hypothyroid, like many human patients with IPEX. The thyroid and pancreatic phenotypes of these mice, like human patients with IPEX, may be variable. Thyroid hormone replacement did not change gonadotropin expression in $Foxp3^{sf/Y}$ mice, leading us to conclude that their infertility is not due to hypothyroidism.

Hypothyroidism is normally accompanied by increased PRL levels. This is because the lack of negative feedback from thyroid hormone causes an increase in hypothalamic thyroid-releasing hormone, which is a secretagogue for PRL [35]. In contrast, we observed reduced expression of *Prl* in $Foxp3^{sf/Y}$ mice [13]. This could mean that a prolactin inhibitory factor is being produced at high levels in $Foxp3^{sf/Y}$ mice.

Taken together, these data suggest that reduced gonadotropin levels are responsible for the arrest in spermatogenesis observed in $Foxp3^{sf/Y}$ mice. In the absence of *Foxp3*, pituitary gonadotropin expression decreases, resulting in hypogonadotropic hypogonadism and infertility. This hypogonadotropic hypogonadism is a secondary effect, most likely due to loss of *Foxp3* in immune cells. Thus, loss of *Foxp3*, likely in regulatory T cells, results in hypogonadotropic hypogonadism and infertility.

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