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

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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 autoimmune 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 scurvy (sf), results in an IPEX-like syndrome in mice. This mutation has been mapped to the Foxp3 gene [10]. Interestingly, affected males (Foxp3sf/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 Foxp3sf/Y male mice [13]. In the following studies we characterize the gonadal phenotype in Foxp3sf/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. Foxp3sf/Y females were mated to Foxp3sf/Y males to obtain Foxp3sf/Y male offspring. Foxp3sf/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

Foxp3sf/Y mice and Foxp3+Y littermates were injected with 5 IU of human chorionic gonadotropin (hCG) starting at Postnatal Day 28 (P28) or P14.
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: 48°C for 15 min, 95°C 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<sub>T</sub> 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 Inc.) and Foxp3 (Mm20352933E; Applied Biosystems by Life Technologies Inc.) were 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.

**Thyroid Treatment**

Purina Test Diets provided thyroid hormone-enriched mouse chow consisting of special pelletedAIN-76A diet with thyroid gland powder added at a concentration of 25 mg/kg chow. Foxp3<sup>-/-</sup> female mice were mated to C57BL/6J males. The date the copulatory plug was detected was considered to be Embryonic Day 0.5. Pregnant Foxp3<sup>-/-</sup> female mice were fed either thyroid chow or control chow ad libitum starting at Embryonic Day 16.5. Foxp3<sup>-/-/y</sup> 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<sup>-/-</sup> and Foxp3<sup>+/+</sup> 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 (**).
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 Foxp3sf/Y mice treated with 5 IU of hCG for 4 wk. Histological analysis of testis sections revealed that testis from Foxp3sf/Y mice is very similar to that of their Foxp3+/Y littermates (Fig. 4, F–H). Thus, spermatogenesis in Foxp3sf/Y 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 Foxp3sf/Y mice, suggesting that like many humans with IPEX, Foxp3sf/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 Foxp3sf/Y mice, pregnant Foxp3sf/+ dams were fed chow containing thyroid powder or a control chow beginning at Embryonic Day 16.5 (Fig. 5A). Foxp3sf/Y pups remained with their dams and continued to receive their respective diets until 6 wk of age, when tissues were collected. Foxp3sf/Y mice

FIG. 1. A) Foxp3 mRNA is detected in the testis at 6 wk of age. B) Reproductive tracts from Foxp3+/Y and Foxp3sf/Y mice at 6 wk of age. Foxp3sf/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 × 10⁻³ ± 0.09 × 10⁻³ vs. 0.82 × 10⁻³ ± 0.09 × 10⁻³), and seminal vesicle weight/body weight (E; 4.26 × 10⁻³ ± 0.25 × 10⁻³ vs. 0.31 × 10⁻³ ± 0.17 × 10⁻³) at 6 wk of age. F) Testicular testosterone content is significantly reduced in Foxp3sf/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 Foxp3sf/Y and Foxp3+/Y littermates (*P < 0.05, **P < 0.01).
that were fed thyroid chow exhibited a very significant reduction in \textit{Tshb} expression, consistent with the reversal of hypothyroidism (Fig. 5B). No significant change in \textit{Fshb} or \textit{Lhb} expression levels occurred when \textit{Foxp3sf/Y} mice were fed thyroid chow, indicating that hypothyroidism is not the cause of the infertility seen in \textit{Foxp3sf/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**

\textit{Foxp3} \textsuperscript{sf/Y} Male Mice Are Hypogonadal and Infertile

\textit{Foxp3} mutant males are infertile and exhibit reduced gonadotropin expression [12, 13]. Although \textit{Foxp3} transcript is present in the testis, FOXP3 protein is not detected. The testicular phenotype of \textit{Foxp3} \textsuperscript{sf/Y} mice is very similar to that of mice lacking gonadotropins. \textit{Hpg} mice are gonadotropin deficient because of a spontaneous mutation in the \textit{Gnrh} gene [20, 21]. Treatment of \textit{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 \textit{Lhb} (\textit{Lhb}) or the receptor for LH (\textit{Lhcgr}) are hypogonadal and infertile, with reduced testosterone levels, resulting in spermatogenesis being arrested at the round spermatid stage [23–25]. The similarity between \textit{Foxp3} \textsuperscript{sf/Y} mice and \textit{Lhb} and \textit{Lhcgr} null mice, combined with the rescue of spermatogenesis in \textit{Foxp3} \textsuperscript{sf/Y} mice by activating LH signaling, suggests that the gonadal phenotype in \textit{Foxp3} \textsuperscript{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, \textit{Foxp3} \textsuperscript{sf/Y} mutant males become fertile, suggesting that their infertility is secondary to autoimmunity [12]. Nude mice have an autosomal recessive mutation of \textit{Foxn1}, causing them to be athymic and immunosuppressed [26]. Godfrey et al. [14] bred scurfy mice onto a nude mouse background to create \textit{Foxp3} \textsuperscript{sf/Y,Foxn1nu/nu} mice. These mice no longer exhibited autoimmunity. They did exhibit immunosuppression consistent with that observed in
Foxn1nu/nu mice. Interestingly, Foxp3sf/Y,Foxn1nu/nu mice were capable of siring progeny. This allowed for generation of Foxp3sf/sf,Foxn1+/nu female mice, which exhibited scurfylke 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 Foxp3sf/Y mice by breeding them onto a nude mouse background rescues their fertility, suggests that the reproductive phenotype in Foxp3sf/Y mice is secondary due to loss of Foxp3 in regulatory T cells.

Foxp3sf/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

**FIG. 3.** A) Foxp3sf/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/Y mouse treated with vehicle (B), Foxp3sf/Y mouse treated with vehicle (C), and Foxp3sf/Y mouse treated with 5 IU of hCG (D) are shown. Original magnification ×400; bar = 100 μm. E) Average testis weight/body weight of Foxp3sf/Y mice is 3.63 × 10^{-3} ± 0.08 × 10^{-3} (data not shown). Tests weights are increased significantly in Foxp3sf/Y mice treated with 5 IU of hCG (1.55 × 10^{-3} ± 0.05 × 10^{-3}) compared with Foxp3sf/Y mice treated with vehicle (0.56 × 10^{-3} ± 0.06 × 10^{-3}). F) Average seminal vesicle weight/body weight of Foxp3sf/Y mice is 2.98 × 10^{-3} ± 0.34 × 10^{-3} (data not shown). Seminal vesicle weights are significantly increased with 5 IU of hCG (3.06 × 10^{-3} ± 0.14 × 10^{-3}) compared with Foxp3sf/Y mice treated with vehicle (0.10 × 10^{-3} ± 0.02 × 10^{-3}). G) Average testicular testosterone levels for Foxp3sf/Y mice treated with vehicle are 7.13 ± 4.12 ng per testis (data not shown). Testicular testosterone levels increased significantly in Foxp3sf/Y mice treated with 5 IU of hCG for 2 wk (8.81 ± 3.27 ng per testis) compared with Foxp3sf/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 Foxp3sf/Y mice treated with hCG or vehicle (*P < 0.05, **P < 0.01).
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 Foxp3sf/Y mice is due to cytokine inhibition of gonadotropin expression or GnRH release.
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 Foxp3sf/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 Foxp3sf/Y mice. Unfortunately, they did not examine thyroid tissue from Foxp3sf/Y mice [34]. We find that Foxp3sf/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 Foxp3sf/Y mice [13]. We 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 thyrotropin-releasing hormone, which is a secretagogue for PRL [35]. In contrast, we observed reduced expression of Prl in Foxp3sf/Y mice [13]. This could mean that a prolactin inhibitory factor is being produced at high levels in Foxp3sf/Y mice.

Taken together, these data suggest that reduced gonadotropin levels are responsible for the arrest in spermatogenesis observed in Foxp3sf/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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REFERENCES


